

⑫

EUROPEAN PATENT APPLICATION

⑮ Application number: **85309454.8**

⑯ Date of filing: **23.12.85**

⑥ Int. Cl.: **C 12 N 15/00, A 61 K 39/21,**
C 12 P 21/02, C 07 K 13/00,
C 12 Q 1/70, G 01 N 33/569,
C 12 N 1/20, C 12 N 5/00,
C 12 N 9/10

③ Priority: **24.12.84 US 685272**
04.12.85

⑦ Applicant: **GENENTECH, INC., 460 Point San Bruno**
Boulevard, South San Francisco California 94080 (US)

④ Date of publication of application: **09.07.86**
Bulletin 86/28

⑦ Inventor: **Capon, Daniel Jeffrey, 817 Oregon Avenue,**
San Mateo California 94402 (US)
Inventor: **Lasky, Laurence Allan, 795 Pacheco Street,**
San Francisco California 94116 (US)

④ Designated Contracting States: **AT BE CH DE FR GB IT**
LI LU NL SE

⑦ Representative: **Armitage, Ian Michael et al, MEWBURN**
ELLIS & CO. 2/3 Cursitor Street, London EC4A 1BQ (GB)

⑤ **Molecularly cloned acquired immunodeficiency syndrome polypeptides, intermediates therefor and methods and materials for their use.**

⑤ Diagnostic product and vaccine for Acquired Immuno-deficiency Syndrome (AIDS) and methods for making and using same, wherein viral polypeptide sequences from an AIDS associated retrovirus are expressed directly or as a fusion polypeptide in a prokaryotic or mammalian cell expression host to produce a diagnostic product which specifically binds complementary antibody produced by individuals afflicted with AIDS or a vaccine against AIDS which confers resistance to infection by AIDS associated retrovirus. The reverse transcriptase of an AIDS associated retrovirus is used separately or in a whole cell assay to identify compounds which selectively inhibit retroviral reverse transcriptase.

EP 0 187 041 A1

MOLECULARLY CLONED ACQUIRED IMMUNODEFICIENCY
SYNDROME POLYPEPTIDES, INTERMEDIATES THEREFOR
AND METHODS AND MATERIALS FOR THEIR USE

5

The present invention relates to immunological products derived from molecular cloning, and to their method of use. More particularly, this invention relates to immunological polypeptides useful as
10 diagnostic products and vaccines in the detection of and vaccination against viral etiological agents of acquired immunodeficiency syndrome. This invention also relates to polypeptides of AIDS retrovirus reverse transcriptase and to their method of use.

15 Analysis of the immune response to a variety of viral infectious agents has been limited by the fact that it has often proved difficult to culture such pathogens in quantities sufficient to permit the isolation of viral polypeptides which may be used (a) as a diagnostic product to detect an immune response to such pathogens or (b) as a
20 vaccine to confer resistance to infection by such pathogens.

The advent of molecular cloning has overcome some of these limitations by providing a means whereby gene products from pathogenic agents can be expressed in virtually unlimited quantities in a non-pathogenic
25 form.

Although antigens from the viruses for influenza (1), foot and mouth disease (2), hepatitis (3), vesicular stomatitis virus and (4), rabies (5), have been reported to be expressed in E. coli, several laboratories have
5 reported that the surface antigen for hepatitis B expressed in prokaryotes is not immunologically reactive with antisera to the naturally occurring antigen (6).

Acquired immunodeficiency syndrome (hereinafter referred
10 to as AIDS) is a devastating disease of the adult immune system which significantly affects cell-mediated immunity. The disease is manifested by a profound lymphopenia which appears to be the result of a loss of T-lymphocytes that have the helper/inducer phenotype T4
15 as defined by the monoclonal antibody OKT4 (7). Other clinical manifestations include opportunistic infections, predominantly Pneumocystis carinii pneumonia, and Kaposi's sarcoma (7).

20 Pre-AIDS, a syndrome that often precedes the onset of AIDS, is characterized by chronic generalized lymphadenopathy. It has been reported that about 10% of patients with pre-AIDS develop AIDS (8).

25 The predominant risk group for AIDS includes homosexual and bisexual males, intravenous drug abusers, recipients of blood components (primarily hemophiliacs receiving treatment with Factor VIII) and recipients of blood transfusions (7). The epidemiology of this syndrome
30 indicates that AIDS is caused by an infectious agent which is transmitted by intimate contact or contact with blood or blood products (7).

Recent studies have suggested that a human retrovirus related to the previously described human T-lymphotropic viruses HTLV-I and HTLV-II, may be the causative agent for AIDS (8, 9, 10). Several laboratories have recently
5 isolated and propagated retrovirus associated with AIDS infection. Gallo has identified a virus designated HTLV-III which has been isolated from AIDS patients (11) and propagated in a permissive T-cell line (12). Similarly, Luc Montagnier of the Pasteur Institute in Paris
10 has found a virus in AIDS patients designated LAV (lymphadenopathy associated virus). Some authors have reported that LAV may be similar or identical to HTLV-III (13, 14). More recently, a virus isolated from an AIDS patient, designated ARV (Aids Related Virus), has been
15 identified and cloned (15). These and other AIDS retroviruses are hereinafter referred to as AIDS associated retrovirus.

HTLV-III is believed to be a retrovirus with a genome
20 comprising approximately 10kb of RNA. The virus particle contains a capsid consisting of a number of proteins. The primary core protein is designated p-24 and has a corresponding molecular weight of about 24,000 daltons. The p-24 core protein is synthesized in vivo as part of a
25 precursor polypeptide encoded by the gag region of the HTLV-III genome. This precursor polypeptide is processed in the infected cell to form p-24 and other viral proteins. In addition, an envelope protein designated gp-41 (MW 41,000 daltons), is also a constituent of the virus
30 particle and is encoded in the env region of the HTLV-III genome.

Antibodies to the envelope protein pp-41 of HTLV-III have been detected in serum from AIDS and pre-AIDS patients. Approximately 88% of AIDS and 79% pre-AIDS patients have detectable antibodies to HTLV-III envelope protein (16).
5 One author has reported that there is a 90-100% sero-conversion to such antibodies (8). In addition to antibodies to the gp-41 envelope protein, antibodies to the HTLV-III core protein p-24, and the HTLV-III proteins designated p-55, p-60 as well as the glycoproteins gp-65,
10 gp-120 and gp-160 have been detected in patients with AIDS (16, 17). The detection of antibodies to these and other as yet unknown antigens is, therefore, a significant indication of exposure to or productive infection by the agent responsible for AIDS.

15

In addition, certain of these viral polypeptide sequences may be used as a vaccine to induce the production of neutralizing antibodies conferring resistance to AIDS infection. A need exists for variant sequences such as
20 fusions of the viral polypeptide with highly immunogenic polypeptides, in order to facilitate induction of a high titer immune response as well as deletions of undesired viral sequences.

25 Accordingly, it is an object herein to express, in a prokaryotic host, viral antigens of an AIDS associated retrovirus which are non-pathogenic and which may be used as diagnostic product to detect AIDS.

30 It is a further object of the present invention to prokaryotically express a diagnostic product to detect

AIDS consisting of variant composite polypeptides of naturally occurring AIDS related polypeptide sequences.

Further, an object of the present invention is to express AIDS
5 associated polypeptides or variants, including fusion polypeptides
of an AIDS associated polypeptide which may be used as a vaccine
against AIDS.

Still further, an object of the present invention is to express AIDS
10 RNA dependent DNA polymerase (reverse transcriptase) for use in an
assay to identify transcriptase inhibitors.

The entire genome of an AIDS-associated retrovirus has been cloned
15 and sequenced. In accordance with this invention, DNA encoding
AIDS-associated polypeptides is identified and employed in
recombinant prokaryotic or mammalian cell culture systems to
synthesize predetermined AIDS-associated polypeptides and
derivatives and amino amino acid sequence variants thereof.
20 Derivatives of AIDS-associated polypeptides include unglycosylated
or variantly glycosylated polypeptides, as well as formylmethionyl
N-terminal species.

Variants of normal AIDS-associated virus polypeptides are provided
25 wherein one or more amino acid residues of the normal polypeptides
have been deleted or substituted by other residues, or one or more
amino acid residues inserted. These variants include predetermined
fragments of normal polypeptides (deletion variants), fusion
polypeptides containing an AIDS-associated virus polypeptide or
30 fragment thereof with a second polypeptide which is not
AIDS-associated virus polypeptide or fragment thereof (fusion or

insertional variants), and all of the above in which an amino residue has been substituted.

DNA encoding the polypeptides of this invention are provided, as
5 well as vectors operably incorporating such DNA and mammalian or prokaryotic cell cultures transformed therewith.

The predetermined polypeptide sequences of an AIDS-associated retrovirus produced herein are essentially free of other naturally
10 occurring AIDS related polypeptides. These polypeptides contain at least one antigenic determinant which is capable of specifically binding complementary antibody. Specific polypeptide sequences described herein are designated p-24, p-15, E', reverse transcriptase and envelope (env) polypeptides. In preferred
15 embodiments, fragments and/or fusions of predetermined polypeptide sequences of an AIDS associated retrovirus, containing at least one antigenic determinant capable of specifically binding complementary antibody, are provided. In one species of this embodiment, a C-terminal portion of the p-24 and the entire p-15 sequence is
20 removed. This truncated p-24, expressed in E. coli, demonstrates an unexpected reactivity with antibody to an AIDS associated retrovirus. Particularly preferred fragments are those of the env protein gp120 which are able to bind normal host cell receptors in competition with the intact virus, as well as E' polypeptide
25 fragments.

The fusion polypeptides comprise (a) a predetermined polypeptide sequence of an AIDS associated retrovirus or fragment thereof having at least one antigenic determinant capable of specifically binding
30 complementary antibody and (b) a second polypeptide sequence which is not immunologically reactive with antibodies normally present in a biologically derived sample which is to be assayed for the

presence of antibodies to AIDS associated retrovirus. If the fusion polypeptide is to be used as a component of a vaccine the second polypeptide sequence also should be incapable of inducing antibodies which are cross-reactive with polypeptides which are naturally
5 occurring in the subject such vaccine is directed to. In this case, polypeptides from lower eukaryotes other than from yeast are useful.

In one species of a fusion polypeptide, DNA sequences encoding a polypeptide sequence of human growth hormone (HGH) are positioned
10 five prime to a DNA sequence encoding the p-24 and p-15 polypeptide sequence of HTLV-III. This is expressed and processed in E. coli as an HGH-p24 fusion polypeptide which specifically binds complementary antibody. Similarly, fusions of E' and env polypeptides such as gp41 and gp120 with viral, synthetic or prokaryotic polypeptides are
15 provided. These results demonstrate that viral antigens associated with AIDS may be expressed as a fusion polypeptide to detect naturally occurring antibodies produced by AIDS infected individuals.

20 The polypeptide sequences of this invention are formulated into therapeutically effective dosages with a pharmaceutically acceptable vehicle and administered to AIDS-susceptible animals in order to induce the production of antibodies to such polypeptide sequences.

25 In a contemplated embodiment, a polypeptide comprising or consisting of a predetermined polypeptide sequence of RNA dependent DNA polymerase (reverse transcriptase) from an AIDS associated retrovirus is cloned and expressed for use in an assay system to identify compounds which inhibit such AIDS associated reverse
30 transcriptase. Such compounds may be administered as a pharmaceutical agent to inhibit infection by AIDS associated retrovirus or dissemination of such retrovirus in infected individuals.

Brief Description of the Drawings

Figure 1 shows the amino terminal polypeptide sequence of p-24 core protein obtained from live HTLV-III retrovirus and the corresponding DNA sequence deduced therefrom.

5

Figures 2a-2d show the DNA sequence, the putative polypeptide sequence and a partial restriction map of the HTLV-III genome. The terminal repeat ("R") and unique regions (U5 for 5' and U3 for 3') are shown. The putative positions assigned to the gag region, reverse transcriptase ("pol"), envelope ("env") and E' and P' proteins are shown. The designations sd and sa are splice donor and splice acceptor sites, respectively. Overlapping amino acid sequences represent putative mRNA splicing phenomena. Alternative bases indicate differences found among clones, indicative of variation found among strains in the viral population.

10

Figure 2e depicts a restriction enzyme map of the provirus DNA and overlapping cDNA clones.

Figure 2f shows the putative mRNA splicing activity of the virus, the resulting messengers and the proteins thereby encoded.

Figure 3 depicts the strategy employed to subclone p7.11.

Figure 4 depicts the construction of p-24 and truncated p-24 expression vectors.

Figure 5 depicts the construction of expression vectors for a p-24 composite polypeptide and a truncated p-24 composite polypeptide.

30

Figure 6 is a Western Blot of the polypeptide expressed by pDE-24, pDE24ΔHD3, pGHp-24ΔB and pGHp-24ΔHD3. The blot was treated with rabbit anti-HTLV-III antibody.

Figure 7 is a Western blot of the polypeptide expressed by pDE-24. The blot was treated with human serum derived from an individual with AIDS.

5 Figures 8a, 9a and 10a depict the structure of the plasmids ptrpLE.gp41, ptrpLE.E' and pSVE.E'DHFR. These plasmids are used for the expression, respectively, of fusions of the env and the E' protein of HTLV-III in bacteria and of the unfused E' protein in CHO cell culture.

10 Figures 8b, 9b and 10b show polyacrylamide gel electrophoresis patterns demonstrating the synthesis, respectively, of the protein fusions encoded by ptrpLE.gp41, ptrpLE.E' and unfused E' protein encoded by pSVE.E'DHFR.

15 Figure 11a shows the region of the HTLV-III genome used for env protein preparation in recombinant higher eukaryotic cell culture.

Figure 11b depicts an expression plasmid for secreting an HTLV-III
20 envelope protein from cultured mammalian cell culture transformants.

Detailed Description

Applicants have demonstrated that viral protein from an
AIDS associated retrovirus can be expressed directly or
5 as a variant polypeptide in host cells and that such
recombinant polypeptides are capable of specifically
binding antibody to an AIDS associated retrovirus. Such
variant polypeptides include viral polypeptides fused
with one or more second polypeptide sequences as well as
10 deletions, insertions, substitutions and derivatives of
the viral polypeptide. In addition, directly expressed
and certain variant polypeptides, each of which contain
fragments of a predetermined polypeptide sequence of an
AIDS associated retrovirus, also react with antibody to
15 AIDS associated retrovirus. These results indicate that
such recombinant polypeptides may be used as diagnostic
agents to detect AIDS in individuals, donated blood and
blood products.

20 Further, such polypeptides may be used as immunogens to
induce the production of neutralizing antibodies which
confer resistance to infection by an AIDS associated
retrovirus.

25 Still further, the reverse transcriptase of an AIDS
associated retrovirus may be used to identify compounds
which may inhibit infection by AIDS associated retrovirus
or the dissemination of such retrovirus in infected
individuals.

30 The fusion polypeptides of the present invention com-
prise an AIDS associated polypeptide sequence and a

second polypeptide sequence. These second polypeptide sequences may be used to: 1) promote secretion of the fusion polypeptide from a bacterial host into the extra-cellular environment or the periplasm of gram negative bacteria, 2) facilitate the functional association of the fusion polypeptide with the surface membrane of recombinant host cells or 3) provide a polypeptide sequence which may be used to purify the fusion polypeptide (e.g. purification of an HGH-AIDS fusion polypeptide by fractionation on an immunoadsorbent specific for HGH). Depending upon the particular applications, second polypeptide sequences used to form a fusion polypeptide with an AIDS associated retrovirus may be of prokaryotic or eukaryotic origin and may be positioned at the amino terminus, carboxy terminus, at both ends of the AIDS associated polypeptide sequence, or inserted within the AIDS associated polypeptide sequence.

Examples of second polypeptide sequences which may be used to promote secretion of the fusion polypeptide include (1) the signal sequence of Herpes Simplex Virus gD protein disclosed in copending U.S. application 527,917 of 29 August 1983 (EP 139417); (2) the signal sequence of E. coli alkaline phosphatase or E. coli enterotoxin STII disclosed in copending U.S. application 658,342 of 5 October 1984 (EPA 85307044.9), and references disclosed therein, and (3) pre-HGH disclosed in copending U.S. application 488,232 of 25 April 1983 (EP 127305) or other higher eukaryotic signal sequences such as that of gamma interferon.

An example of a second polypeptide sequence which facilitates functional membrane association is the

transmembrane sequence of Herpes Simplex Virus disclosed in U.S. application 527,917 filed August 29, 1983 (EP 139417).

5 Since many individuals at risk for AIDS also have antibodies to E. coli and other enterobacteria, the second polypeptide must be chosen to avoid false positive immunological reactivity with these antibodies. Polypeptide sequences from enterobacteria should therefore be used as a second polypeptide only if such sequences are removed during processing or otherwise prevented from reacting with
10 the biologically derived samples to be assayed for the presence of antibody produced in response to infection by an AIDS associated virus, e.g. by recombinant expression such that the bacterial protein epitopes are modified so as to no longer be cross-reactive with the native protein (see the LE fusions described below).

15 When the fusion polypeptide of the present invention is used as a vaccine against AIDS infection, the second polypeptide sequence must be chosen to avoid the production of antibodies to polypeptides which are naturally occurring in the subject such vaccine is directed to.
20 For example, in a vaccine for humans the second polypeptide sequence is preferably not HGH. Such vaccines, however, may contain prokaryotic polypeptide sequences or preferably eukaryotic polypeptide sequences other than those of yeast and primates.

25 The present invention specifically discloses the cloning and expression of certain HTLV-III-encoded polypeptides. However, the present invention also contemplates the cloning and expression of other HTLV-III polypeptides. HTLV-III polypeptides which possess antigenic determinants to antibodies for AIDS and pre-AIDS patents
30 include gp-160,

gp-120, gp-65, gp-41, p-60/p-55. The gp-120 polypeptide appears to be a precursor polypeptide for gp-65 and gp-41. These particular HTLV-III polypeptides are illustrative and are not intended to limit the scope of the invention.

In addition, the present invention contemplates the generation of a library of products, each containing different antigenic determinants that may be used to determine which antigenic determinants are best suited for detection of AIDS or pre-AIDS. Such a library, for example, may be used to determine which antigenic determinants are immunologically reactive to serum derived from healthy individuals who are serologically positive for AIDS. Those antigenic determinants which test positive to such serum but negative to serum from AIDS patients may be prime candidates for a vaccine to induce the production of neutralizing antibodies. Further, diagnostic products containing such antigenic determinants may be used to identify individuals with neutralizing antibodies who are unlikely to develop the severe clinical manifestations associated with AIDS.

Although the present invention is based on studies of HTLV-III it is to be understood that HTLV-III may be similar or identical to LAV or ARV. As so related, polypeptide products derived from those retroviruses are within the scope of the present invention. Accordingly, the designation AIDS associated retrovirus refers to HTLV-III, LAV, ARV and/or other retrovirus that may cause AIDS or pre-AIDS.

As used herein, a polypeptide sequence of an AIDS associated retrovirus is the full length native polypeptide sequence or the predetermined sequence derived from genomic sequencing.

5

A naturally occurring (native) polypeptide sequence is the polypeptide formed in virus infected cells or found in the culture fluid of such cells.

10 Variant polypeptide sequences of an AIDS associated retrovirus include: (1) fusions of viral polypeptide or fragments thereof with second polypeptide sequences including N and C terminal fusions and insertions; (2)
15 deletions of the N-terminal, C-terminal, or an internal region of the polypeptide sequence of viral polypeptide to produce a fragment of a polypeptide sequence of an AIDS associated retrovirus; (3) substitutions of one or
20 more amino acids in a polypeptide sequence of an AIDS associated retrovirus and (4) derivatives such as labeled or bound viral polypeptide sequences which may be labelled by well known techniques or bound to a solid phase such as that disclosed in U.S. patent 3,720,760.

25 "Second polypeptides" are sequences which are fused with a polypeptide sequence of an AIDS associated retrovirus or fragment thereof to form the fusion polypeptide sequences of the present invention. These second polypeptides may be full length or partial protein sequences
30 of eukaryotic, non-AIDS viral or prokaryotic origin and may be used to promote secretion of the fusion polypeptide, facilitate association of the fusion polypeptide with the surface

membrane of an expression host or aid in the purification of the fusion polypeptide. When used as a vaccine, the second polypeptide of a fusion polypeptide is a sequence which is not normally capable of inducing antibodies
5 which are cross-reactive with naturally occurring polypeptides, in the subject such vaccine is directed to.

"Complementary antibody" refers to antibody raised against a corresponding naturally occurring viral
10 epitope or epitope encoded by AIDS-associated retrovirus.

A DNA sequence of an AIDS associated retrovirus encodes the polypeptide and variant polypeptide sequences of the present invention described above.

15

"Biologically derived sample" includes any biological fluid or tissue sample taken from a human or animal subject which may be assayed to detect the presence of complementary antibody produced in response to exposure
20 to or infection by an AIDS associated retrovirus. Such samples typically comprise blood, urine, semen, and saliva but may include any biological material in which such complementary antibody or AIDS associated retrovirus may be found.

25

Prokaryotes are preferred for cloning and expressing DNA sequences to produce the diagnostic product and vaccine of the present invention. For example, E. coli K12 strain 294 (ATCC No. 31446) is particularly useful.
30 Other microbial strains which may be used include E. coli strains such as E. coli B, and E. coli X1776 (ATCC No. 31537), and E. coli c600 and c600hf1, E. coli W3110 (F⁻,

λ^- , prototrophic, ATTC No. 27325), bacilli such as Bacillus subtilus, and other enterobacteriaceae such as Salmonella typhimurium or Serratia marcesans, and various pseudomonas species. When expressed in prokaryotes the polypeptides of the present invention typically contain an N-terminal methionine or a formyl methionine, and are not glucosylated. These examples are, of course, intended to be illustrative rather than limiting.

10 In general, plasmid vectors containing replication and control sequences which are derived from species compatible with the host cell are used in connection with these hosts. The vector ordinarily carries a replication site, as well as sequences which encode proteins that are capable of providing phenotypic selection in transformed cells. For example, E. coli is typically transformed using pBR322, a plasmid derived from an E. coli species (18). Plasmid pBR322 contains genes for ampicillin and tetracycline resistance and thus provides easy means for identifying and selecting transformed cells. The pBR322 plasmid, or microbial plasmid must also contain, or be modified to contain, promoters which can be used by the microbial organism for an expression of its own proteins. Those promoters most commonly used in recombinant DNA construction include β -lactamase (penicillinase) and lactose promoter systems (19-21) and a tryptophan (trp) promoter system (22, 23). While these are the most commonly used, other microbial promoters have been discovered and utilized, and details concerning the their nucleotide sequences have been published, enabling a skilled worker to ligate them functionally with plasmid vectors (24). In the specific embodiments disclosed, a

trp promoter (22, 23) was used to express the diagnostic product and vaccine of the present invention.

In addition to prokaryotes, eukaryotic cells may be used
5 to express the AIDS associated virus polypeptides including particularly the reverse transcriptase of an AIDS associated retrovirus. Saccharomyces cerevisiae, or common baker's yeast is the most commonly used among eukaryotic microorganisms, although a number of other
10 strains are commonly available. For expression in Saccharomyces, the plasmid YRp7, for example, (25-27) is commonly used. The plasmid already contains the trp1 gene which provides a selection marker for a mutant strain of yeast lacking the ability to grow in
15 tryptophan, for example ATCC No. 44076 or PEP4-1 (28). The presence of the trp1 lesion as a characteristic of the yeast host cell genome then provides an effective environment for detecting transformation by growth in the absence of tryptophan.

20

Suitable promoting sequences in yeast vectors include the promoters for 3-phosphoglycerate kinase (29) or other glycolytic enzymes (30, 31), such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase,
25 pyruvate, decarboxylase, phosphofructokinase, glucose-6-phosphate, isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase. In constructing suitable expression plasmids, the termination
30 sequences associated with these genes are also ligated into the expression vector 3' of the sequence desired to be expressed to provide polyadenylation of the mRNA

termination. Other promoters, which have the additional advantage of the transcription controlled by growth conditions are the promoter regions for alcohol dehydrogenase 2, isocytochrome C, acid phosphatase, degradative enzymes associated with nitrogen metabolism, and the aforementioned glyceraldehyde-3-phosphate dehydrogenase, and the enzymes responsible for maltose and galactose utilization. Any plasmid vector containing yeast-compatible promoter, origin of replication and termination sequences is suitable.

10 Cultures of cells derived from multicellular organisms also are employed for expression of AIDS associated retrovirus proteins. Mammalian or vertebrate cells are of particular interest, such as VERO and HELA cells, Chinese Hamster ovary (CHO) cell lines, and WI38, BHK, COS-7 and MDCK cell lines. Expression vectors for such
15 cells ordinarily include an origin of replication, a promoter for controlling expression of the DNA encoding the AIDS associated retroviral polypeptide, along with a mammalian selection marker, RNA splice site, polyadenylation site and transcriptional terminator sequences as required.

20 Vectors capable of transforming mammalian host cells to expression of AIDS associated polypeptides are preferably introduced into host cells with a selection marker, e.g. the gene encoding DHFR (dihydrofolate reductase) in known fashion and then amplified by
25 exposing the transformants to increasing concentrations of selection agent, e.g. methotrexate. For example see U.S. patent 4,399,216.

For use in mammalian cells, the transcriptional and translational control functions are conventionally obtained from viral sources.
30 For example, commonly used promoters are derived from polyoma, Simian Virus 40 (SV40) and most particularly Adenovirus 2. The early and late promoters of SV40 virus are useful as is the major late promoter of adenovirus as described above. Further, it is also possible, and often desirable, to utilize promoter or control sequences normally
35 associated with

the desired gene sequence, provided such control sequences are compatible with the host cell systems.

5 An origin of replication may be provided either by construction of the vector to include an exogenous origin, such as may be derived from adenovirus or other viral (e.g. Polyoma, SV40, VSV, BPV, etc.) source, or may be provided by the host cell chromosomal replication mechanism, if the vector is integrated into the host cell
10 chromosome.

For vectors of the invention which comprise DNA sequences encoding both AIDS associated polypeptide and a cotransformation, selection and amplification gene such
15 as the DHFR enzyme, it is appropriate to select the host according to the type of DHFR protein employed. If wild type DHFR protein is employed, it is preferable to select a host cell which is deficient in DHFR, thus permitting the use of the DHFR coding sequence as a marker for
20 successful transfection in selective medium which lacks hypoxanthine, glycine, and thymidine.

On the other hand, if DHFR protein with low binding affinity for MTX is used as the controlling sequence, it
25 is not necessary to use DHFR resistant cells. Because the mutant DHFR is resistant to methotrexate, MTX containing media can be used as means of selection provided that host cells are themselves methotrexate sensitive.

30 Alternatively, a wild type DHFR gene may be employed as an amplification marker in a host cell which is not

deficient in DHFR provided that a second drug selectable marker is employed, such as neomycin resistance.

An example, which is set forth hereinafter, contemplates the use of
5 CHO cells as host cells and an expression vector which encodes the reverse transcriptase of an AIDS associated retrovirus.

As more fully set out below, the diagnostic product of the present invention is utilized in place of its counterpart derived from a live
10 pathogen in analogous immunoassays. In that regard, a commercial diagnostic test kit would include the above diagnostic products with a variety of other immunological products, at least one of which is labeled, for detection of its complementary antibody or the antigen. The system has been described with respect to the molecular cloning
15 and expression of specific proteins of HTLV-III which possess sufficient antigenic determinants to render them capable of specifically binding complementary antibody, namely antibody to HTLV-III. The specific techniques for cloning and expressing exemplary polypeptides are set forth in more detail in the examples
20 that follow.

There are a number of known techniques for the determination of an unknown quantity of antigen or antibody in biological fluids such as serum, urine, or saliva or from skin samples or the like. In
25 principle, the present invention utilizes such known techniques but substitutes certain molecularly cloned diagnostic reagents of a type set forth above in the otherwise known procedure.

Accordingly, the procedures themselves will be described only generally with reference being made to conventional immunology text for the details of the procedures. It would be well known to skilled workers in the field how
5 to utilize the novel diagnostic products of the present invention in conventional immunological techniques.

For simplicity of description, the general term "diagnostic product" will be used in describing the antigen
10 functional product of the present invention. The term "diagnostic product" is defined as a predetermined polypeptide sequence of an AIDS associated retrovirus with one or more antigenic determinants capable of specifically binding complementary antibody induced by a
15 AIDS associated retrovirus. The diagnostic product is formed in a recombinant host cell capable of its production. The polypeptide sequence may be either functionally associated with a surface membrane of the recombinant cell or it may be recovered and used free of
20 the host cell membrane. Further, the antigenic polypeptide sequence may be fused to a second polypeptide sequence.

The diagnostic methods used in assaying AIDS associated
25 retrovirus, its constituent polypeptides and complementary antibodies are conventional. These include the competitive, sandwich and steric inhibition techniques. The first two methods employ a phase separation step as an integral part of the method while
30 steric inhibition assays are conducted in a single reaction mixture. The methodology for assay of retrovirus or its polypeptides on the one hand and for

substances that bind retrovirus or viral polypeptides on the other hand are essentially the same, although certain methods will be favored depending upon the size of the substance being assayed. Therefore the substance to be
5 tested is referred to herein as an analyte, irrespective of its status otherwise as an antigen or antibody, and proteins which bind to the analyte are denominated binding partners, whether they be antibodies, cell surface receptors or antigens.

10 Analytical methods for AIDS associated retrovirus, its polypeptides, complementary antibody or cell surface receptors all use one or more of the following reagents: Labelled analyte analogue, immobilized analyte analogue,
15 labelled binding partner, immobilized binding partner and steric conjugates. The labelled reagents also are known as "tracers".

The label used is any detectable functionality which does
20 not interfere with the binding of analyte and its binding partner. Numerous labels are known for use in immuno assay, examples including enzymes such as horseradish peroxidase, radioisotopes such as ^{14}C and ^{131}I , fluorophores such as rare earth chelates or fluorescein,
25 spin labels and the like. Conventional methods are available to covalently bind these labels to proteins or polypeptides. Such bonding methods are suitable for use with AIDS associated retrovirus, viral polypeptides, complementary antibody and retrovirus receptors, all of
30 which are proteinaceous.

Immobilization of reagents is required for certain assay methods. Immobilization entails separating the binding partner from any analyte which remains free in solution. This conventionally is accomplished by either
5 insolubilizing the binding partner or analyte analogue before the assay procedure, such as by adsorption to a water insoluble matrix or surface (Bennich et al., U.S. 3,720,760) or by covalent coupling (for example using glutaraldehyde cross-linking), or by insolubilizing the
10 partner or analogue afterward, e.g., by immunoprecipitation.

Steric conjugates are used in the steric hinderance method for homogeneous assay. These conjugates are
15 synthesized by covalently linking a low molecular weight hapten to a small analyte so that antibody to hapten substantially is unable to bind the conjugate at the same time as anti-analyte. Under this assay procedure the analyte present in the test sample will bind
20 anti-analyte, thereby allowing anti-hapten to bind the conjugate resulting in a change in fluorescence when the the hapten is a fluorophore.

Other assay methods, known as competitive or sandwich
25 assays, are well established and widely used in the commercial diagnostics industry.

Competitive assays rely on the ability of a labelled analogue (the "tracer") to compete with the test sample
30 analyte for a limited number of binding sites on a common binding partner. The binding partner is generally insolubilized before or after the competition and then

the tracer and analyte bound to the binding partner are separated from the unbound tracer and analyte. This separation is accomplished by decanting (where the binding partner was preinsolubilized) or by centrifuging (where the binding partner was precipitated after the competitive reaction). The amount of test sample analyte is inversely proportional to the amount of bound tracer as measured by the amount of marker substance. Dose-response curves with known amounts of analyte are prepared and compared with the test results in order to quantitatively determine the amount of AIDS associated retrovirus, viral polypeptide or complementary antibody present in the test sample. These heterologous assays are called ELISA systems when enzymes are used as the detectable markers.

Another species of competitive assay is a homogenous assay which does not require a phase separation. Here, a conjugate of an enzyme with the analyte is prepared so that when anti-analyte binds to the analyte the presence of the anti-analyte modifies the enzyme activity. In this case, a polypeptide of an AIDS associated retrovirus or its immunologically active fragments are conjugated with a bifunctional organic bridge to an enzyme such as peroxidase. Conjugates are selected for use with complementary antibody so that binding of the complementary antibody inhibits or potentiates enzyme activity. This method per se is widely practiced under the name EMIT.

Sandwich assays particularly are useful for the determination of polypeptides of an AIDS associated retrovirus, complementary antibody or retrovirus cell

surface receptors, i.e., large molecules. In sequential sandwich assays an immobilized binding partner is used to adsorb test sample analyte, the test sample is removed by washing, the bound analyte is used to adsorb labelled
5 binding partner and bound material then separated from residual tracer. The amount of bound tracer is directly proportional to test sample analyte. In a "simultaneous" sandwich assay, test sample is not separated before adding the labelled binding partner.

10 The foregoing are merely exemplary assays for AIDS associated retrovirus, polypeptides of an AIDS associated retrovirus, complementary antibody and retrovirus cell surface receptors. Other methods now or hereafter
15 developed for the determination of these analytes are included within the scope hereof.

In order to simplify the examples certain frequently
20 occurring and well-known methods employed in recombinant constructions will be referenced by shorthand phrases or designations.

Plasmids are generally designated by a lower case p
25 preceded and/or followed by capital letters and/or numbers. The starting plasmids or sources of DNA herein are commercially available, are publicly available on a restricted basis, or can be constructed from available plasmids or polynucleotides in accord with published
30 procedures. In addition, other equivalent plasmids are known in the art and will be apparent to the ordinary artisan since the plasmids generally only function as

replication vehicles for the preprotein and its control sequences, or for elements thereof in intermediate constructions.

5 "Digestion" of DNA refers to catalytic cleavage of the DNA with an enzyme that acts only at certain locations in the DNA. Such enzymes are called restriction enzymes, and the sites for which each is specific is called a restriction site.

10

The various restriction enzymes used herein are commercially available and their reaction conditions, cofactors and other requirements as established by the enzyme suppliers were used. Restriction enzymes commonly
15 are designated by abbreviations composed of a capital letter followed by other letters and then, generally, a number representing the microorganism from which each restriction enzyme originally was obtained. In general, about 1 µg or plasmid or DNA fragment is used with about 1
20 unit of enzyme in about 20 µl of buffer solution. Appropriate buffers and substrate amounts for particular restriction enzymes are specified by the manufacturer. Incubation times of about 1 hour at 37°C are ordinarily used, but may vary in accordance with the supplier's
25 instructions. After incubation, protein is removed by extraction with phenol and chloroform, and the digested nucleic acid is recovered with aqueous fraction by precipitation with ethanol. Digestion with a restriction enzyme infrequently is followed with bacterial alkaline
30 phosphatase hydrolysis of the terminal 5' phosphates to prevent the two restriction cleaved ends of a DNA fragment from "circularizing" or forming a closed loop upon

ligation (described below) that would impede insertion of another DNA fragment at the restriction site. Unless otherwise stated, digestion of plasmids is not followed by 5' terminal dephosphorylation. Procedures and
5 reagents for dephosphorylation are conventional (32).

"Recovery" or "isolation" of a given fragment of DNA from a restriction digest means separation of the digest by polyacrylamide gel electrophoresis, identification of the
10 fragment of interest by comparison of its mobility versus that of marker DNA fragments of known molecular weight, removal of the gel section containing the desired fragment, and separation of the DNA from the gel, generally by electroelution. This procedure is known generally.

15 A "Western Blot" is a method by which the presence of polypeptide is confirmed by reaction with labelled complementary antibody. The polypeptide is separated electrophoretically on a polyacrylamide gel and
20 electrophoretically transferred to nitrocellulose. The nitrocellulose is incubated with labelled complementary antibody, unbound antibody removed and the location of residual label is identified.

25 "Transformation" means introducing DNA into an organism so that the DNA is replicable, either as an extrachromosomal element or chromosomal integrant. Unless otherwise provided, the method used herein is the CaCl_2 transformation method (33).

30 "Ligation" refers to the process of forming phosphodiester bonds between two double stranded nucleic

fragments (34). Unless otherwise provided, ligation may be accomplished using known buffers and conditions with 10 units of T4 DNA ligase ("ligase") per 0.5 μ g of approximately equimolar amounts of the DNA fragments to be ligated.

"Fill in" refers to repair of sticky ended (overhanging) restriction enzyme fragments. Generally 2-15 μ g of DNA are incubated in 50 mM NaCl, 10 mM Tris(pH 7.5), 10mM MgCl₂, 1mM dithiothreitol with 250 mM of each of four deoxynucleoside triphosphates and 8 units DNA polymerase Klenow fragment at 24°C for 30 minutes. The reaction is terminated by phenol and chloroform extraction and ethanol precipitation.

"Preparation" of DNA from transformants means isolating plasmid DNA from microbial culture. Unless otherwise provided, the alkaline/SDS method was used (34).

"Oligonucleotides" are short length single or double stranded polydeoxynucleotides which are made by chemically known methods and then purified on polyacrylamide gels (35).

The following specific examples are intended to illustrate more fully the nature of the present invention without acting as a limitation upon its scope.

Example 1

This example discloses plasmid construction and expression of the p-24 core polypeptide of HTLV-III in a
5 prokaryotic expression host.

A. Virus Growth and Viral RNA Isolation

HTLV-III_B/H9 cells (ATCC CRL 8543) were obtained from Dr.
10 Larry Arthur, Frederick Cancer Research Facility, Frederick, MD. Cultures were maintained in suspension and adapted to RPMI 1640 supplemented with 5% FBS, 2 mM L-glutamine, and 100 ug/ml penicillin-streptomycin mixture. Cells were seeded at a density of 3×10^5 viable
15 cells/ml in 10-liter volumes, and cultures harvested at a density of $10-15 \times 10^5$ viable cells/ml approximately 96 hours after seeding.

Following clarification to remove cells, culture fluids
20 were concentrated by ultrafiltration. HTLV-III virus particles were purified by banding of ultra-filtrate concentrate in a 20% to 60% sucrose gradient in a Beckman SW27 rotor for 16hr at 27,000 rpm using a Beckman L8-M ultracentrifuge. Sucrose fractions between 30% and 40%
25 were pooled, diluted with TNE (10mM tris pH 7.5, 0.1M NaCl, 1mM EDTA) and pelleted by differential ultracentrifugation in a Beckman Type 50 rotor. Virus particles were resuspended in TNE at a concentration approximately 2000 times greater than that of the original
30 medium. These concentrates contained 2.5 to 3.0 mg of protein per ml.

Total RNA was extracted from HTLV-III₃/H9 cells (berger, et al., "Biochemistry" 18:5143 (1979)). PolyA containing RNA was purified by fractionation on oligo dT cellulose (Aviv, et al., "P.N.A.S. U.S.A." 74:5463 (1977)) and used
5 for cDNA synthesis.

B. Cloning of the p-24 gene

DNA Synthesis

10 PolyA RNA was primed with oligo dT for reverse transcriptase mediated cDNA synthesis (Capon, et al., "Nature" 304:507 (1983)). Second strand DNA synthesis by DNA polymerase I was self-primed. The resulting doubled
15 stranded DNA was treated with S1 nuclease to nick the resultant loop and to remove single stranded nucleotides. This blunt ended double stranded DNA was used to construct an HTLV-III phage library.

20 Phage Library Construction and
Detection of HTLV-III Clones

The above double stranded DNA was blunt end ligated with T-4 ligase to R-1 linkers (37) which were synthesized by known techniques (35). This R-I/cDNA was ligated into
25 Eco R1 digested λ GT10 to generate an HTLV-III cDNA library as disclosed (37).

Two copies of the phage library were replica plated onto nitrocellulose filters where the DNA was fixed for in
30 situ hybridization (32). One set of filters was hybridized with ³²P labelled cDNA prepared from the polyA positive RNA obtained from HTLV-III infected T-cells

which was used to generate the cDNA library. The other set of filters was hybridized with ^{32}P labelled cDNA obtained from the same cell line which was not infected with HTLV-III. Differential phage hybridization, analogous to the method employed for detecting bacterial cDNA clones (38), allowed applicants to identify a number of HTLV-III positive clones.

Subcloning and Sequencing of of HTLV-III Clones

10

The above-identified HTLV-III clones from the cDNA phage library were digested with Eco RI. The cDNA inserts were isolated and subcloned into the RI site of pBR322 (ATCC 37017) and propagated in E. coli 294 (ATCC 31446).

15

A number of these subcloned RI inserts were sequenced by the dideoxy method (39), producing the proviral sequence shown in Figs. 2a-2d.

20

Amino Terminal Sequencing

Live HTLV-III virus particles were purified as previously described. The constituent proteins were fractionated on a 12% polyacrylamide gel using Laemmli buffer (42). Approximately 600 ug of virus protein was loaded per lane under reducing conditions (41). The gel was stained with Coomassie blue and a protein band having a corresponding molecular weight of 24,000 daltons was cut from the gel. The protein was electroeluted from the gel (41) for amino terminal sequence analysis (42). The amino acid sequence for the first 17 amino acids, excluding residue 16, is shown in Figure 1. The corresponding

25

30

DNA sequence is shown directly below the amino acid sequence. A comparison of this DNA sequence with that obtained for the HTLV-III genome in Figure 2 revealed one particular clone, designated p 7.11 in Figure 3, which
5 contained a DNA sequence encoding this peptide sequence. This approximately 2.2 kilobase covers the precursor gag region and encodes, 5' to 3', p-12, p-15, p-24 a second p-15 protein, and approximately 300 extra base pairs 3' to the gag region. The DNA sequence and corresponding
10 amino acid sequence of this precursor gag region is shown in Figure 2. A partial restriction map of the 220 amino acid p-24 core protein region including an R-1 site 3' from the expected carboxy terminal amino acid is depicted for p-7.11 in Figure 3.

15

Subcloning of p 7.11

Plasmid 7.11 was digested with Eco R1. The 2.2 kilobase insert was isolated and double digested with RsaI and
20 PstI. The RsaI-PstI and PstI-EcoR1 sequences were isolated. Plasmid UC9 (43) was digested with SmaI and Eco R1. The large fragment comprising most of pUC9 was isolated and hybridized with the Rsa-I PstI and PstI-Eco-R1 fragments. The blunt RsaI and Sma I ends
25 were ligated with T-4 ligase. The resulting plasmid, designated pUCp-24, was used to transform E. coli 294.

In constructing pUCp-24 the RsaI site of p-7.11 was used. In so doing, the first ten N-terminal amino acids were
30 excluded from this construction. To incorporate these ten amino acids a synthetic oligonucleotide was constructed (35). The DNA sequence of this 39-mer is

Plasmid pSRCex16 (44) contains pBR322 and a fusion of the first 69 nucleotides of HGH with the DNA encoding the 60,000 MW phosphoprotein of SRC, the fusion being under the control of the E. coli trp promoter. It is not
30 important that the plasmid encode any protein. Instead, any pBR322 plasmid derivative which contains a trp promoter is satisfactory (45).

Plasmid pSRCex 16 is partially digested with EcoRI and filled using the Klenow fragment of DNA polymerase I in order to destroy the EcoRI site in the trp promoter, thereby leaving an EcoRI site at the beginning of the HGH
5 fragment. The resulting plasmid, pEARI5RCex16 was then digested with EcoRI in order to open the plasmid, trimmed with SI nuclease to blunt the ends and ligated to a linker (fsl-2) (46) having the following structure-

10 5' pAATTATGGAATTCCAT 3'
 TTAATACCTTAAGGTAp

Ligation with the fsl-2 linker recreates the EcoRI site immediately after the ATG, which is underlined above, at
15 the EcoRI cleavage point indicated by an arrow. Accordingly, the first base of the first codon of p24/p-15, as constructed below, is provided by the vector. This plasmid is designated pHGH-SRC-fsl-2.

20 Construction of a Full Length p-24
 Expression Vector

Plasmid UCp24L was cleaved with BamHI. The cleaved fragment was treated the Klenow fragment of DNA polymerase I in the presence of 0.5mM nucleotide
25 triphosphates to fill in the BamHI restriction site. This fragment was then digested with EcoRI. The fragment containing the p-24 and p-15 (p-24/p-15) DNA sequence was isolated.

30 Plasmid HGH-SRC-fsl-2 was cleaved with EcoRI and filled in as previously described. The cleaved plasmid was subsequently digested with BamHI. The fragment

containing the trp promoter and ATG initiation codon was isolated. This fragment was hybridized with a fragment conferring tetracycline resistance derived from digesting pBR322 by digestion with BamHI and EcoRI and with the
5 blunt ended BamHI-Eco-RI fragment obtained from pUCp24L. The blunt ends were ligated with T-4 ligase. This plasmid, designated p24DE was used to to transform E.coli 294. Transformants were selected by their ability to resist tetracycline. Expression of this plasmid was
10 expected to produce a polypeptide consisting of the full length p-24 core protein and the p-15 protein.

Construction of a
p-24 Fragment Expression Vector

15 Plasmid p-24DE contains a HindIII restriction site located in the vicinity of the DNA sequence encoding amino acids 178-179 of the p-24 core protein. In addition, the p-24DE contains a HindIII site in the tetracycline resistance gene derived from pBR322 located 3' to
20 the p-24 DNA sequence. Digestion of p-24DE with HindIII followed by removal of the fragment encoding amino acids 179-200 and the p-15 protein and the religation of the fragment containing the 5' p-24 DNA sequence under control of the trp promoter produced the plasmid designated p-DE-24 Δ HD3. A TAA stop codon within the pBR322
25 sequence 3' to the truncated p-24 DNA sequence results in the expression of the first 178 amino acids of p-24 core protein and eight amino acid residues encoded by the pBR322 DNA sequence.

Example 2

This example discloses the construction and cloning of an expression vector of p-24 core protein sequence of HTLV-III as a fusion polypeptide in a prokaryotic host.

A. Expression Vectors for Fusion Polypeptides of p-24 Core Protein and Truncated p-24 Core Protein

The construction of vectors for the expression of composite polypeptides for the p-24 and a fragment of p-24 are depicted in Figure 5.

Construction of a p-24 fusion polypeptide expression vector

Plasmid UCp24L was cleaved with BamHI, blunt ended and digested with EcoRI as described above. The fragment containing the p-24/pl5 DNA sequences was isolated.

Plasmid HGH 207-1 (45) was digested with PstI and Pvu2. The sequence encoding HGH 3' from the PstI site to the PvuII site was isolated. This sequence excludes DNA sequences 5' from the PstI site as well as the last 8 amino acid residues of mature HGH. These two fragments were hybridized to a PstI-EcoRI fragment derived from pBR322 which contains a gene conferring tetracycline resistance. T-4 ligase was used to ligate the PvuII and blunt ended BamHI site prior to transformation of E. coli 294 with this plasmid, designated pHGH p-24-A.

Plasmid HGHp24-A does not encode the mature HGH amino acid sequence 5' to the Pvu-2 site of pHGH 3'R. A DNA

sequence encoding such residues was inserted into the plasmid by the following method.

Plasmid HGH 3'R was digested with BglIII and ClaI. The
5 fragment containing the tac promoter and HGH 5' DNA sequences encoding mature HGH to the BglIII site was isolated.

Plasmid HGH 207-1 was digested with BglIII and PstI. The
10 BglIII-PstI fragment containing part of HGH and part of p-24 was isolated. The same plasmid was digested with PstI and ClaI followed by isolation of the fragment containing p-15 and part of p-24.

15 These three isolated fragments were ligated to produce pHGHp24-B. This plasmid contains DNA sequences under control of the tac promoter for expression of mature HGH, except for the C-terminal 8 amino acids of HGH, fused to p-24 core protein and p-15 protein.

20

Construction of a Truncated p-24
Fusion Polypeptide Expression Vector

Digestion of pHGHp-24B with HindIII followed by re-ligation results in the same deletion as that obtained
25 for p24DE. The resulting sequence, however, was expected to express a fusion polypeptide of mature HGH (absent 9 C-terminal amino acids) fused to the first 178 amino acids of p-24 and eight amino acid residues from the expression plasmid 3' to the p-24 DNA sequence. This
30 plasmid is designated pHGHp-24 Δ HD3.

Example 2a

This Example describes the isolation of cDNA encoding the gp41 envelope protein, its tailoring for expression in prokaryotes, expression of a p41 fusion in E. coli and the in vitro resolubilization of the expressed fusion.

Viral mRNA was isolated, cDNA prepared from the viral mRNA, and the cDNA cloned into λ GT10 phage as described in Example 1 A and B above. A cDNA clone, H9c.53 was identified that comprised cDNA encoding the complete p41 envelope region (bp 7336 to 8992, Fig. 2d). H9c.53 was digested with RsaI and HindIII and the fragment I corresponding to nucleotides 7417-7722 of the proviral genome was isolated. Fragment I encodes amino acid residues being ValGlnAlaArg and the C-terminal residues being AsnTyrThrSer). Fragment I was ligated to an excess of synthetic EcoRI adaptor (having the sequence AATTCATGTCTTACGGTCAAGG) with a phosphorylated blunt end and a 5' hydroxyl EcoRI end as described above in Example 1B, digested with HindIII in order to cleave ligation-formed dimers, and the resulting 326 bp EcoRI-HindIII fragment isolated on a 6% polyacrylamide gel. Plasmid pNCV(55a) was digested with EcoRI and HindIII and the vector fragment recovered. The 326 bp, gp41-encoding fragment was ligated to the pNCV fragment and the ligation mixture used to transform E. coli (ATCC 31446) to tetracycline resistance. A tetracycline resistant plasmid was recovered (ptrpLEgp41). This plasmid is shown in Fig. 8A. The open reading frame under the control of the trp promoter encodes a gp41 fusion having at its amino terminus 190 residues representing the combined sequence of the amino terminus

of the E. coli trp E gene product and its leader together with the amino acid sequence encoded by the EcoRI adaptor, and at its c-terminus 14 residues encoded by residual pBR322 sequence. The fusion contained 306
5 residues in total.

While the gp41 fusion represents a combined amino and carboxyl fusion of prokaryotic peptides with a gp41 fragment, it will be appreciated that, either or both of
10 the prokaryotic sequences may be deleted or substituted for by other prokaryotic, eukaryotic or synthetic polypeptides. When direct expression of the DNA encoding the gp41 fragment was attempted, rather than as an amino terminal fusion, immunoreactive material
15 could not be identified in the cell culture extracts. The reasons for this are unknown, but may involve instability of the unfused product in E. coli. Accordingly gp41 or its fragments should be expressed in prokaryotes as N-terminal fusions. Such fusions of
20 course will include fusions with the secretory leaders of E. coli periplasmic or extracellular proteins, e.g. the ST-II heat stable enterotoxin or alkaline phosphatase signals, among others. In this case gp41 will be secreted as a mature protein free of prokaryotic
25 sequences. Plasmids encoding fusions of signal peptides with the mature gp41 fragment are readily constructed by inserting synthetic DNA encoding the known sequences of prokaryotic signals in place of the trp LE-adaptor sequence in pgp41 using appropriate flanking
30 restriction sites and adaptors or linkers as required. Any remaining extraneous DNA is excised using M13 phage deletional mutagenesis or other conventional method.

-40-

The gp42 DNA was truncated to the 102 residue fragment so as to avoid including sequences near the amino and carboxyl terminus that are particularly rich in hydrophobic residues, specifically the two long hydrophobic stretches of 28 and 22 amino acids contained within
5 the putative transmembrane region of gp41. Hydrophobic regions are known in some instances to be deleterious to E. coli growth. (56, 57). The region extending from residues 29-130 relative to the amino terminus of gp41 did not contain these hydrophobic regions. Note, however, that other host cells such as mammalian cells are
10 suitable for expression of the intact gp41 protein or gp41 fragments containing one or both terminal hydrophobic regions.

E. coli (ATCC 31446) was transformed with ptrpLEgp41 and grown in M9 minimal media until values at A_{550} of 6.6, 9, 12, 17 and 21 were
15 reached. Total cellular proteins were electrophoretically separated on 10% SDS polyacrylamide gel and stained with Coomassie blue. The results are shown in Fig. 8b, wherein lanes a-e correspond to the above densities in ascending order, while lane f depicts total cellular protein from a pBR322-transformed control (k represents
20 1,000 daltons). E. coli transformants accumulated a prominent protein (LEgp41, at about 10% by weight of the total intracellular bacterial protein) with an apparent molecular weight of 33,000 daltons, consistent with the predicted size of 306 amino acids. Recombinant LEgp41 was purified from refractile bodies enriched for
25 the protein by gel filtration and ion-exchanged chromatography using procedures similar to

those previously described for trpLE-foot and mouth disease virus polypeptide fusions (55a). Specifically, cells were washed in 10mM Tris-HCl, 1mM EDTA, resuspended in 4 volumes of the same buffer, sonicated, and the
5 refractile bodies collected by centrifugation at 5,000 rpm for 30 minutes in a Corvall GSA rotor. The particles were washed twice and solubilized with 7M guanidine HCl, 0.1% β -mercaptoethanol (BME). The resulting extract was clarified at 19,000 rpm for 4 hours in a Beckman Ti60
10 rotor. The high speed supernatant was passed through a Sephacryl S-300 (Pharmacia) column. Fractions from the included volume containing LEgp41 were identified by electrophoresis on SDS-polyacrylamide gels, pooled and dialysed extensively against 7.5M urea, 0.1% BME. This
15 pool was chromatographed on DEAE-52 cellulose (Whatman) in 7.5M urea, 0.1% BME, 10mM Tris-HCl pH 8.5. LEgp41 material that flowed through the column was pooled and dialysed into 4M urea, 10mM Tris HCl pH 8.5 at a concentration of 0.2 mgs/ml. The purified LEgp41
20 appeared greater than 95% homogeneous on SDS-polyacrylamide gels by silver staining. LEgp41 was not glycosylated.

This purification procedure eliminated the need to
25 preadsorb patient sera with E. coli proteins where the sera is destined for assay of its anti-AIDS antibody level using the LEgp41 fusion; apparently there are little or no pre-existing antibodies to the LE portion in patient sera, at least no antibodies in the conformation
30 in which they exist in LEgp41. This also suggests that immunization programs using fusions of bacterial proteins such as LE with HTLV-III sequences presents a low risk of

inducing a hyperimmune response, notwithstanding the fact that humans are commonly exposed to such proteins. Prokaryotic protein fusions for use in vaccines are identified by screening pooled sera from normal
5 individuals for the presence of antibodies that cross-react with the candidate fusion. Conventional methods such as Western Blotting or radioimmunoassay are used for the screening, for example as shown below in Example 2b.

10

Example 2b

Enzyme-linked immunosorbent assay (ELISA)
for the detection of antibodies to LEgp41

15

Purified LEgp41 (Example 2a) was coated in 96-well microtiter plates in 0.05 M sodium carbonate pH 9.6 as described (58). Sera were diluted 1:100 fold in TBS and incubated in coated microtiter wells for 1 hour at room
20 temperature. Following 3 washes with TBS the plates were incubated for two hours at room temperature with antibody-enzyme conjugate (mouse monoclonal anti-human IgG Fc-horseradish peroxidase, Travenol-Genentech Diagnostics). Plates were again washed with TBS,
25 developed with 3,3',5,5'-tetramethylbenzidine, and color development measured in a plate reader (Dynatech) at 590 nm. Samples with absorbance values greater than twice the mean of negative control sera were scored as positive.

30

Significant antibody levels to purified LEgp41 were detected in sera from 125 of 127 (98%) clinically

diagnosed AIDS patients. By comparison, none of 300 sera from random blood donors previously shown to be seronegative for HTLV-III using a commercial whole virus ELISA test kit were found to react with LEgp41 above the
5 cutoff absorbancy ($OD_{590} > 0.12$). The two AIDS patient sera which did not react with LEgp41 were the only AIDS sera nonreactive by commercial whole virus ELISA. One of these sera was found to react with whole virus on Western Blots but this reactivity could be attributable to p24
10 core antigen. The other LEgp41 negative AIDS sera did not react with virus on Western Blots, although virus could be cultured from this patient. The reactivity of LEgp41 with the 125 positive AIDS sera was highly sensitive, ranging from 0.21-1.64 OD_{590} units, with only
15 3 sera giving OD_{590} values of < 0.4 units. Thus, recombinant LEgp41 shows excellent specificity and sensitivity in detecting antibodies directed against the AIDS retrovirus.

20 The purified LEgp41 ELISA similarly detected antibodies found in sera from ARC (AIDS-related complex) patients and healthy homosexual men. Sixty of 69 (86%) ARC patient sera reacted with LEgp41, yielding essentially identical results as the commercial whole virus ELISA.
25 Of the 9 nonreactive ARC samples, Western blot analysis with whole virus revealed that two of the sera were positive for p24 only, while the remaining seven sera were completely nonreactive although virus could be recovered from four of these patients.

30 As expected, a small fraction of sera obtained from asymptomatic homosexual men (26/75, 34%) was reactive in

the LEgp41 ELISA. The 26 LEgp41 ELISA-positive sera from this cohort included all 24 positive sera detected by the commercial whole virus ELISA and were confirmed positive by Western Blots utilizing whole virus. Significantly,
5 two of the LEgp41 seropositive samples from healthy gay men were found to be repeatably nonreactive in the commercial whole virus ELISA.

There was no evidence of nonspecific reactivity in the
10 LEgp41 ELISA with sera from six patients with diseases that characteristically give false-positive responses in other serological tests (systematic lupus erythematosus, rheumatoid arthritis, heterophile positive mononucleosis or Goodpasture's syndrome). As an additional test of
15 specificity, sera previously giving false-positive reactions in a commercial whole virus ELISA were examined by the LEgp41 ELISA. A group of 58 sera which had given nonrepeatable positive results with the commercial whole virus ELISA were found not to react in the LEgp41 ELISA.
20 Furthermore, 25 sera which gave repeatable positive results in the commercial ELISA test, but which were negative in more accurate Western Blot assays, also did not react in the LEgp41 ELISA.

25 Example 3

Immunoassay of expressed recombinant polypeptide

A. Expression of full length and truncated p-24 and p-24 fusion polypeptide

30 E. coli 294 containing each of the Example 2 plasmids is first grown in L broth which is rich

in tryptophan. This medium represses the trp promoter. Expression of recombinant polypeptide is induced by transferring the culture to M-9 medium, which does not contain tryptophan, after the L-broth culture has obtained an O.D of approximately 0.5 at 550 nm. After about one hour, indole acetic acid is added to the medium to produce a final concentration of about 10ug/ml to further induce expression. After an additional 5-6 hours at 37°C the cells are collected by centrifugation, lysed (47) and subjected to Western blot analysis.

B. Immunological Assay of Expressed Polypeptides

Rabbit Anti-HTLV-III

15

The polypeptides expressed by the above-identified plasmids were assayed for immunological reactivity with serum from rabbits inoculated with Triton treated HTLV-III retrovirus and which were subsequently boosted with live HTLV-III virus.

A Western blot of p24DE and p24DEΔHD3 polypeptides and pHGHP24B and pHGHP24ΔHD3 fusion polypeptides expressed in M-9 medium supplemented with indole acetic acid and reacted with HTLV-III rabbit antiserum and ¹²⁵I labelled Protein A is shown in Figure 6. A number of immunologically reactive bands for the p24DE polypeptide are apparent in lane 3. The band corresponding to a molecular weight of about 44,000 daltons corresponds to an expressed protein comprising p-24 and p-15. Surprisingly, two doublets with molecular weights of approximately 24,000 and 15,000 were also observed. The

cause of these doublet signals is not presently understood. However, the detection of these doublet signals with molecular weights which are consistent with the gag proteins produced naturally from the polycistronic gag region indicate that this strain of E. coli is capable of processing such precursor polypeptide sequences to produce HTLV-III proteins which may be detected by complementary antibody. A Western blot of p-24DEΔHD3 polypeptide is also shown in lane 4 of Figure 6. Again, a doublet having a molecular weight of about 20,000 daltons is observed. The absence of a 44,000 dalton p-24/p-15 full length HTLV-III protein and the 15,000 dalton doublet detected for p24DE is consistent with the above interpretation of the p24DE Western blot.

Lanes 5 and 6 of Figure 6 contain respectively the immunologically reactive proteins expressed by pHGHp24B and pHGHp24ΔHD3. The band in lane 5 having a molecular weight of approximately 65,000 daltons is consistent with the expected composite polypeptide constructed. Further, the band in lane 6 has the expected molecular weight of about 45,000.

Assay with Human AIDS Serum

The polypeptide expressed by plasmid p24DE was assayed for immunological reactivity with serum from an individual afflicted with AIDS. In Figure 7, strip 3 depicts the Western blot obtained from p24DE polypeptide treated with human AIDS serum and ¹²⁵I labelled Protein A. A diffuse band having a molecular weight of approximately 24,000 daltons can be seen. Strip 2 is a control

which contains p-24DE polypeptide treated with normal human serum and ¹²⁵I labelled protein A. Strip 1 contains size markers. This assay indicates that polypeptides of the present invention can be used to detect
5 complementary antibody made in response to exposure to or infection by an AIDS associated retrovirus.

Vaccine

10 The vaccines of the present invention are contemplated and comprise the predetermined polypeptides and fusion polypeptides previously described. The following examples disclose the use of vaccines containing p-24 polypeptide sequences from HTLV-III. However, any polypeptide
15 sequence of an AIDS associated retrovirus, especially those encoded by the env region of the retrovirus genome, may be used.

Example 3

20

Immunization of Mice

The polypeptides encoded by p-24DE and p-24DEΔHD3 are used to immunize BALB/C mice. Each mouse is immunized
25 with 5-25 ug of p-24DE or p-24DEΔHD3 polypeptide contained in 200 ul of an emulsion consisting of 50% aqueous antigen and 50% complete Freund's adjuvant. Each mouse is immunized at multiple intradermal and subcutaneous sites as follows: 25 ul in each rear foot
30 pad, 50 ul in the tail and 100 ul distributed among 3-5 intradermal sites along the back. A control group is similarly injected with emulsion which does not contain

antigen. Four weeks after primary immunization the mice are boosted with 5-25 ug of polypeptides as above with the exception that the emulsion was prepared with incomplete Freund's adjuvant. For the booster
5 immunization each mouse receives 200 ul of the antigen emulsion or emulsion lacking antigen distributed as follows: 50 ul in the tail and 50 ul distributed among intradermal sites along the back. Three weeks after
10 boosting approximately 500 ul of blood is collected from each mouse by tail bleeding. The sera obtained from this blood is used for in vitro neutralization studies.

In Vitro Neutralization Studies

15 Sera from mice immunized with p-24DE or p-24DEΔHD3 polypeptide and from mice in the control group are tested for the ability to neutralize HTLV-III in vitro. 25 ul of serially diluted mouse serum (2-fold dilution: 1:8 to 1:16384) is incubated with 175ul of HTLV-III concentrate
20 for one hour at 37° in Dulbecco's modified Eagle medium. After incubation, each dilution is applied to approximately 40,000 HUT-78 T-cells (48) contained in each well of a 96 well tissue culture plate. After 3-4 days incubation, virus growth is determined by an
25 immunofluorescence assay for HTLV-III (49) or by a reverse transcriptase assay. The absence of HTLV-III infection of HUT-78 T-cells indicates that the polypeptide induces the production of neutralizing antibodies to HTLV-III in mice.

Primate Assay for Neutralizing Antibodies

The polypeptides which raise neutralizing antibodies in mice are used to immunize chimpanzees which are known to develop generalized lymphadenopathy when infected by HTLV-III retrovirus. Each chimpanzee in an experimental group is immunized with 50-100 ug of polypeptide contained in a 200 ul emulsion consisting of 50% aqueous antigen and 50% complete Freund's adjuvant. Each chimpanzee is immunized as follows at multiple intradermal sites. A control group is injected with emulsion not containing antigen. After four weeks the chimpanzees in each group are boosted with 200 ul of the emulsion or antigen emulsion as above except that the emulsion is prepared with incomplete Freund's adjuvant.

Five weeks after boosting, the chimpanzees in the experimental and control group are challenged with various doses of HTLV-III concentrate. Those polypeptides which prevent the development of lymphadenopathy in the experimental group of chimpanzees are candidates for a vaccine which is capable of inducing the production of neutralizing antibodies in humans which resist infection by AIDS associated retrovirus.

Because complete Freund's adjuvant is not acceptable for use in humans, the above identified polypeptides which prevent lymphadenopathy in chimpanzees challenged by HTLV-III are formulated with an adjuvant suitable for human use. Such formulation may comprise alum precipitated polypeptide complexes (22) which are used to immunize chimpanzees in an experimental group. A control group is vaccinated with adjuvant alone. Formulations

which prevent lymphadenopathy in the experimental group comprise a polypeptide in admixture with a pharmaceutically acceptable vehicle which may be used as a human vaccine.

5

Example 5

The composite polypeptides encoded by p~pHGH p-24-B and pHGHp24ΔHD3 are used in a manner analogous to that disclosed in Example 4 to determine which composite polypeptide confers resistance to AIDS infection in chimpanzees as evidenced by the absence of the development of lymphadenopathy in immunized chimpanzees challenged with HTLV-III retrovirus. The use of HGH polypeptide sequences in such composite polypeptides, however, is not preferred for a human vaccine against infection by AIDS associated retrovirus since such composite polypeptides may induce an autoimmune response against HGH in human subjects. Accordingly, a composite polypeptide vaccine to resist infection by an AIDS associated retrovirus in humans should consist of a predetermined sequence of an AIDS associated retrovirus or fragment thereof expressed as a fusion polypeptide with a secondary polypeptide sequence which is not capable of inducing a substantial autoimmune response to polypeptides naturally occurring in humans.

AIDS Associated RNA Dependent
DNA Polymerase

30

The polypeptide sequence of an AIDS associated RNA dependent DNA polymerase (AIDS reverse transcriptase) is contemplated to be used in an assay to identify compounds

which inhibit such transcriptase activity and which may be used as a pharmaceutical agent to inhibit infection by AIDS associated retrovirus or dissemination of such retrovirus in infected individuals. Suramin, a known
5 inhibitor of AIDS reverse transcriptase (53), is disclosed in the following example. However, the assay of other compounds for transcriptase inhibition is contemplated because of the severe clinical side effects that Suramin elicits when administered to humans (53).
10 Examples of such selective inhibitors have been disclosed by several laboratories (54).

Example 6

15 A. Cloning and Expression
 of AIDS Reverse Transcriptase

The region of the viral genome encoding the AIDS associated retrovirus reverse transcriptase of HTLV-III
20 terminates at nucleotide 4674. Its N-terminus is located within the region extending from nucleotide 1639 to about nucleotide 1800. For the purposes described below it is preferred to select an amino terminus that is located within the gag region (1639-1769), thereby encompassing
25 all optional amino termini. The region located proximate to nucleotide 1800 includes a number of suitable met codons which are useful to the start codons for constructions expressing the reverse transcriptase. However, it is not critical that in situ met codons be
30 selected. Instead, an exogenous, vector-borne ATG codon is ligated to partial exonuclease digests or M13 deletion mutants of the 5' region of the reverse transcriptase

gene. Constructions that are properly in-frame are easily identified by the ability of transformant cell extracts to reverse transcribe RNA. Alternatively, the normal in vivo N-terminus for the reverse transcriptase
5 as processed in a given host is determined by purifying the enzyme from infected cells, sequencing the amino terminus in accord with methods known per se and identifying the cDNA nucleotide sequence encoding the amino acid sequence of the amino terminus. Preferably,
10 the cDNA of the figures is digested with BglII, which cleaves at nucleotide 1642, and SalI (5367). The 3725 bp fragment is recovered. N-terminal cleavage sites other than BglII are at 1738 (DdeI) and 1754 (AluI). However, these enzymes also cleave at points internal to the
15 reverse transcriptase gene. Thus, it would be necessary to conduct a partial digest with DdeI or AluI in order to expect to recover a full length gene. Another cleavage site other than SalI which is located distal to the reverse transcriptase gene is that of StuI (at 4987).

20

Having isolated DNA encoding the reverse transcriptase gene which bears terminal ligation sites, the DNA is ligated into a replicable vector. The vector will be selected depending upon the intended host, and this in
25 turn on whether the DNA is to be simply replicated or whether it will be desired to use the vector for expression of the enzyme. Ordinarily, the vector will contain a bacterial origin of replication and an antibiotic selection gene, e.g. for tetracycline resistance.
30 These elements are available in a large number of publicly known plasmids such as pBR322. Since it is preferred to express the reverse transcriptase in higher eukaryotic

cells, the vector will also contain elements necessary for the stable replication of the gene, for identification of transformants, for promoting the transcription of the gene and for properly terminating the transcribed mRNA in higher eukaryotes. Typically, these will be respectively, the SV40 origin of replication and a source of T antigen (such as supplied by chromosomal integrants found in such cell lines as COS-1, available from Cold Spring Harbor Laboratories), a gene encoding mouse thymidine kinase, the SV40 early or late promoters and the Hepatitis B surface antigen polyadenylation site. Such vectors are known, for example, see EPA 73,656; 92,182 and 93,619 all of which are incorporated by reference.

Known vectors may not contain convenient restriction sites for direct insertion of the reverse transcriptase DNA obtained as described above. This will not constitute an obstacle to those skilled in the art since methods are known per se for introducing new restriction sites or for converting cohesive-ended restriction termini into blunt ends. For example, the hepatitis surface antigen gene in pHS94 of EPA 73,656 is excised by EcoRI and BamHI digestion, EcoRI and BamHI linkers ligated to the BglII and SalI sites of the cDNA fragment, respectively, and the modified cDNA ligated into the vector fragment obtained from the pHS94 digestion. Then the construction is completed in accord with EPA 73,656.

The vector bearing the reverse transcriptase gene is transfected into permissive hosts such as the COS-1 monkey kidney cell lines or the CHO (Chinese hamster

ovary) cell line. Other stable eukaryotic cell lines may be employed as hosts, as well as yeast and prokaryotes where appropriate origins of replication and promoters are provided (for yeast, the two micron origin and a
5 promoter such as that of metallothionein, and for bacteria the pBR322 origin and a promoter such as trp which is described elsewhere herein in connection with expression of the gag and envelope proteins of the AIDS associated retrovirus in prokaryotes).

10 In this connection, other AIDS associated retroviral proteins such as gag and envelope polypeptides may be expressed in eukaryotic cells, whether yeast or mammalian, as such cells bear a more immediate
15 phylogenetic relationship to the normal retroviral hosts than do prokaryotes.

The reverse transcriptase is preferably synthesized in recombinant culture under the control of an inducible
20 promoter so that any toxic effects of the polymerase on the cell are minimized until a generous amount of mRNA has accumulated. Alternatively, the gene encoding the enzyme is ligated at its 5' end to a signal sequence recognized and processed by the intended host, which in
25 the case of higher eukaryotes, for example, include the known secretion signals for interferons, secreted hormones like insulin or viral surface antigens.

B. Assay of Reverse Transcriptase Inhibition

30 A cell culture expressing AIDS reverse transcriptase is lysed and divided into aliquots. Various amounts of the

compound to be assayed are added to each aliquot of lysate. An oligonucleotide of polyA together with oligo dT primers and ^{32}P labelled TTP is added to each aliquot. After incubation at 37°C , the amount of acid precipitated counts is determined. Those compounds
5 which inhibit AIDS reverse transcriptase but which do not comparatively inhibit human DNA polymerase are then selected for further in vitro toxicity and efficacy studies.

Alternatively, AIDS reverse transcriptase is recovered from an
10 expression host by methods known per se, including immunoaffinity adsorption, Sephadex gel filtration, ion exchange chromatography and electrofocusing on native polyacrylamide gels. The recovered reverse transcriptase is then used to assay compounds as described above.

15

EXAMPLE 7

Cloning and Expression of the E' Polypeptide

An E' polypeptide of AIDS-associated retrovirus is defined as the 206 residue polypeptide designated "E'" in Fig. 2, its naturally-occurring
20 alleles, or its amino acid sequence variants which are immunologically cross-reactive with antisera capable of binding the E' polypeptide produced in cells infected with AIDS-associated retrovirus. In addition to the C-terminal deletional derivatives described below, other deletions, insertions or substitutions that do not substantially
25 change the immunoreactivity of the peptide with sera from patients infected with HTLV-III or other AIDS-associated retro viruses are included within the scope of the term "E' polypeptide" or "E' protein" as used herein. A further example of a deletional variant is E' protein in which the first 19 residues, through Arg₁₉, are deleted.
30 The trpLE fusion described below is an insertional variant. Other variants will be apparent to the ordinary artisan. They are readily produced by methods of recombinant synthesis or, in the case of certain deletions, by proteolytic hydrolysis.

35 In accordance with this invention, E' polypeptides are produced by

recombinant methods so as to be free of proteins from AIDS-associated retrovirus-infected cells that are not encoded by the AIDS-associated retrovirus. Such polypeptides obviously are not present in AIDS-associated retrovirus virions.

5 cdNA clone H9.c53 contained the E' sequence. The E' sequence is shown in Fig. 2 commencing at nucleotide 8375. The first stop codon in reading frame downstream from the ATG at 8375 is an OP stop codon at nucleotide 8993. The Cys residue immediately preceding this stop
10 codon presumably is the C-terminus of the E' polypeptide. However, other C-termini upstream from this Cys residue also are within the scope of this invention. E' polypeptides include sequences that are C-terminated at any residue within about the last 50 residues shown in Fig. 2, preferably immediately adjacent and downstream from a lysinyl
15 or arginyl residue.

An expression plasmid for the synthesis of a fusion of the E' polypeptide of Fig. 2 with a bacterial polypeptide is described hereafter. One aliquot of H9.c53 was digested with HaeIII and XhoI
20 and the 62bp fragment coding for E' residues 14-34 recovered (fragment 1). Another aliquot was digested with XhoI and HindIII and the 719bp fragment was recovered which encodes amino acids 35-206 and contains the stop codon followed by untranslated 3' sequence (fragment 2).

25 A synthetic oligonucleotide (fragment 3) was prepared (35) having the following sequence encoding the first 13 residues of the E' polypeptide flanked at its 5' end by an EcoRI cohesive terminus and ending with a 3' blunt end for ligation to the HaeIII-generated blunt end of fragment 1.

30

PAATTCATGGGTGGCAAGTGGTCAAAAAGTAGTGTGATTGGATGG-OH
HO-GTACCCACCGTTCACCAAGTTTTTCATCACAACCTACCP

pNCV(55a) was digested with EcoRI and HindIII, which are unique sites in pNCV, and the vector fragment recovered. This vector fragment was
35

ligated simultaneously to fragments 1, 2 and 3, the ligation mixture transformed into E. coli 294 and antibiotic resistant colonies identified. ptrpLE-E' was obtained from a resistant colony by preparation of plasmid DNA. The structure of this plasmid is shown in Fig. 9a.

ptrpLE-E' contains a continuous open reading frame encoding a bacterial (E. coli) derived protein (LE) fused at its C-terminus to the full E' sequence.

10 This plasmid was employed as follows to prepare the E' protein fusion (LE-E'). ptrpLE-E' was transformed into E. coli strain 294, grown overnight in LB broth (32) containing 5 mcg/ml tetracycline, diluted 1:50 into M9 broth (32) containing tetracycline and grown at 37°C to
15 an absorbance of 0.5 at 550 nm. Total cell proteins were prepared from 25 ml of induced cell culture. Cells were resuspended in 1/250 volume of 0.01 M Tris-HCl pH7.5, 0.001 M EDTA, 0.03 M mercaptoethanol and 0.8% SDS, boiled for 2 minutes and precipitated with 3 volumes of cold acetone. The precipitated proteins were redissolved by boiling
20 in SDS-polyacrylamide gel loading buffer (40).

Initial attempts to directly express unmodified E' sequences in E. coli were unsuccessful due to an apparent instability of the protein. However, the foregoing method gave efficient expression of the E' polypeptide in E. coli. LE is a protein of 190 residues derived from translated sequences of the trp leader and trpE gene product (55b), which forms stable, insoluble aggregates in vivo and has been used successfully to stabilize synthesis of other foreign proteins in E. coli (55c, 55a). Expression of the LE-E' fusion protein from
25 ptrpLE-E' is under the control of an E. coli trp promoter and trp leader ribosome binding site (Fig. 9a). Cultures of E. coli transformed with ptrpLE-E' were grown under conditions of tryptophan depletion as described above to derepress the trp promoter, and their proteins analysed by electrophoresis on 10% SDS-polyacrylamide gel and
35 staining with Coomassie blue. As shown in Fig. 9b, cells containing

plasmid ptrpLE-E' accumulate a prominent protein of Mr 41,000 (lane b), which is not present in cultures of E. coli transformed with pBR322 (lane c). The Mr 41,000 protein has two important characteristics of the expected LE-E' fusion protein. First, this protein is seroreactive with antisera to LE, and second, it has an Mr of 22,000 greater than LE (Fig. 9b, lane a) consistent with the size predicted for a protein containing 206 additional amino acids.

To determine whether LE-E' exhibits antigenic sites recognized by human sera following exposure to the AIDS retrovirus, total cell proteins extracted from E. coli transformed with ptrpLE-E' were employed as an antigen in a Western blot assay. Note that individual sera were first preadsorbed with a soluble extract of E. coli proteins (blocking extract), although this was later found to be unnecessary.

In the Western blot assay, total cellular proteins were prepared from induced cultures of E. coli transformed with ptrpLE-E' (Fig. 9a) or ptrpLE-gp41 (described above) and electrophoresed on SDS-10% polyacrylamide gels as described above. Proteins were electrophoretically transferred to nitrocellulose sheets as described(55e). Individual blot strips were incubated overnight at room temperature with a 1:200 dilution of the indicated sera in TBS buffer (0.025 M Tris-HCl pH7.2, 0.15 M NaCl and 0.05% Tween-20) containing 5% normal goat serum and 5 mcg/ml protein blocking extract from E. coli transformed with pBR322. Blocking extract was prepared by sonicating bacteria in 0.01 M Tris-HCl pH 7.5, 0.15 M NaCl and 0.5% NP40, and removing the insoluble residue by centrifugation at 10,000xg. Total cell proteins prepared from E. coli transformed with pNCV or ptrpLE-E' were employed for preadsorption of sera with LE or LE-E' proteins, respectively. After extensive washing in TBS buffer, the strips were successively incubated with biotinylated goat anti-human or anti-rabbit IgG (Vector Labs), avidin conjugated horseradish peroxidase (Miles-Yeda) and developed with peroxidase substrate (0.5 ng/ml 4-chloro-1-naphthol and 0.16% hydrogen peroxide in TBS). Following each incubation, strips were washed extensively with TBS.

Sera from 37% of AIDS patients (17/46), 81% of ARC patients (21/26), and 39% of healthy homosexual men (29/75) tested gave a clear reaction with the LE-E' immunoblots, while none of 37 sera from random blood donors was found to react (Table 1 below). Identical results were obtained for a representative subset of these sera in Western blot experiments in which total cell proteins from *E. coli* transformed with plasmid ptrpLE17-E', a derivative of ptrpLE-E', were utilized as the antigen. These cells express LE17-E', a fusion protein that differs from LE-E' in that only residues 1-17 of the LE protein are present, indicating that the seroreactivity detected between AIDS-related sera and LE-E' fusion proteins is specific for epitopes associated with E' sequences.

Table 1
Prevalence of antibodies to bacterial LE-E' in AIDS risk groups

Risk group	<u>E'+</u>	<u>gp41+</u>	<u>E'+/ gp41+</u>	<u>E'+/ gp41-</u>	<u>E'+/ gp41+</u>	<u>E'+/ gp41-</u>	<u>WB+</u>	<u>WB+gp41+</u>
AIDS	17/46	44/46	16/46	1/46	28/46	1/46 ^(a)	45/45	44/46
ARC	21/26	22/26	19/26	2/26	3/26	2/26	22/26	22/26
HHM	29/75	24/75	18/75	11/75	6/75	40/75	24/75	24/75
cntrls	0/37	0/37	0/37	0/37	0/37	0/37	N.D. ^(b)	N.D.

E', reactivity in LE-E' western blot assay; gp41, reactivity in LE-gp41 western blot assay (see Examples above); WB, reactivity in whole virus western blot assay; HHM, healthy homosexual males; controls, random blood donor samples.

- (a) This individual was positive in an HTLV-III cultivation assay
 (b) All 37 control sera were seronegative in a commercial whole virus ELISA (Abbott)

Two major points are evident from this comparison of LE-E' and gp41 seroreactivity in different AIDS risk groups. First, LE-E'

seroreactivity is far less frequently detected than gp41 seroreactivity in AIDS patients (37% vs. 96%), but with roughly the same frequency with sera from ARC patients (81% vs. 85%) and healthy homosexual males (39% vs. 32%). Secondly, a significant number of the healthy homosexual males were seropositive for LE-E' but not gp41 (11/75). The fact that antibodies to E' are detectable sooner than antibodies to env antigens in a statistically significant number (11/75) of healthy homosexual males, a group at high risk for developing AIDS and ARC, as well as a smaller number of AIDS and ARC patients (3/72), was completely unexpected and surprising particularly since no protein of the Mr of E' has been identified as a virion component and, because E' contains no apparent secretory leader, it would not be expected to find the protein in the serum. Seroconversion to env antigens was monitored by Western blots utilizing either whole HTLV-III virus (55e) or a recombinant LE-gp41 antigen (supra), each assay having a reliability of >98% in detecting sera from clinically diagnosed AIDS patients. Since many of the healthy homosexual men studied here were seronegative for E' as well as virus antigens and consequently may not have been exposed to the virus, the actual frequency of E'+gp41- individuals in this sample may be as high as 31% (11/35). These results may be related to previous observations that HTLV-III can be isolated from the lymphocytes of symptom-free seronegative persons (55m), and have considerable practical importance for the early clinical diagnosis of and screening for AIDS retrovirus exposure.

In contrast to the situation found for asymptomatic individuals with virus exposure and ARC patients, antibodies to E' are detected at a much lower frequency than antibodies to gp41env in AIDS patients (37% vs. 96%). A similar pattern of less frequent reactivity among AIDS patients has been previously noted for the core protein p24gag relative to the major envelope protein gp120env in radioimmuno-precipitation analysis (55n). It is unclear whether this phenomenon represents a differential susceptibility to developing AIDS, but more likely reflects the selective loss of antibody titers or lower titers

to E' and p24gag than to viral envelope antigens.

To obtain direct evidence for E' expression in HTLV-III-infected cells, high titer antisera specific for E' were prepared by immunizing rabbits with LE-E' isolated by preparative SDS-polyacrylamide gel electrophoresis. The region of the gel containing LE-E' (about 100 mcg) was excised, homogenized in incomplete Freund's adjuvant and administered subcutaneously at two week intervals. The rabbit sera were screened for the ability to immunoprecipitate E'-related proteins from extracts of HTLV-III-infected cells metabolically labelled with ³⁵S-cysteine. The publicly available H9/HTLV-IIIB cell line was employed for this purpose, since we had previously observed that these cells produce a high level of 1.7-1.9 kb mRNAs potentially capable of encoding an E' translation product (~1% of polyA+ RNA) (55e).

Following four injections of antigen, sera obtained from two rabbits were capable of immunoprecipitating 5-6 distinct proteins of Mr 23,500-28,000 from H9/HTLV-IIIB cells. Preimmune sera from the same animals failed to immunoprecipitate any one of these proteins. Furthermore, the immune sera did not detect any of these proteins in uninfected H9 cells. The proteins detected in H9/HTLV-IIIB cells by the immune sera included three major proteins of Mr 28,000, 25,500 and 25,000 and two minor proteins of Mr 26,500 and 23,500. In some experiments the Mr 23,500 protein could be resolved into two distinguishable species.

LE-E' positive sera from 3 AIDS patients and 1 ARC patient were capable of immunoprecipitating proteins from the HTLV-III infected cells of ~Mr 28,000 and 25,000 that were not precipitated by sera from control donors. The immunoprecipitation of these proteins could be only partially displaced by LE-E' protein, however, suggesting that the LE-E'+ AIDS and ARC sera recognize E' determinant(s) not displayed by the recombinant protein.

This rabbit antiserum contained antibody capable of binding AIDS-associated retroviral E' polypeptide but was free of any antibody

capable of binding any other AIDS-associated retroviral-encoded polypeptide as well being free of bound E' polypeptide. It conventionally is immobilized to facilitate separations. For example, the antiserum or antibody is bound or adsorbed to a polyolefin, e.g. polystyrene microtiter plate wells, or to matrices to which anti-
5 rabbit IgG (e.g. goat) has been preadsorbed.

The E' protein next was expressed in recombinant mammalian cell culture. Applicants' starting plasmid was obtained from pFD11 (EP
10 117,060A) by a complex procedure using certain plasmids conveniently available to applicants. This procedure is not preferred for use by the art. Instead, the starting plasmid is preferably obtained from pFD11 by the following method. pFD11 is digested with HindIII at its unique HindIII site and the vector recovered. An adaptor having

15 the sequence AGCTTGGATCCTTTTATCGATA
 ACCTAGGAAAAATAGCTATTCCA

is ligated to the vector fragment and transformed into E. coli 294. pFD11d is obtained from an ampicillin resistant colony. The ligation of the adaptor into pFD11 introduces BamHI and Clal sites immediately upstream from the mouse DHFR gene. pFD11d is digested with BamHI and
20 Clal and the vector fragment isolated.

H9C.53 was digested with BamHI and TaqI and the fragment (fragment 4) was isolated that contained the portion of the env gene downstream from the BamHI site at nucleotide 8053, the E' coding region and its
25 untranslated 3' region through to nucleotide 232. The untranslated 3' region included the 3' long terminal repeat (LTR). The pFD11d vector fragment (supra) was ligated to fragment 4 and E. coli 294 transfected. pSVE.E'DHFR was obtained from an ampicillin resistant colony. The E'-encoding DNA thus was placed under the transcriptional
30 control of the SV40 early promoter, while the LTR provided sequences for the cleavage and polyadenylation of the E' gene transcripts together with sequences for promoting transcription of the dhfr gene.

35 CHO dhfr-K1 DUX-B11 cells (55f) were grown on DMEM medium containing

10% fetal bovine serum. Transfections with pSVE.E'DHFR were performed by the calcium phosphate precipitation method (55g) as described (55h). Following a 6 hour exposure to the vector, the cells were shocked with 20% glycerol (v/v) in PBS (55i) and grown for 1-1/2 days in nonselective medium. Cells were then passaged into selective medium (F12 medium lacking glycine, hypoxanthine, and thymidine and supplemented with 10% dialysed fetal bovine serum) and refed every 2-3 days. A population of approximately 300 resistant colonies was massed after two weeks. Immunoprecipitation analysis of a population of the initial dhfr+ colonies revealed a barely detectable level of E'. This population was further amplified for pSVE.E'DHFR by selection for growth in methotrexate (55j, 55k). Approximately 2×10^5 cells were seeded into a 100 mm dish of selective medium containing 10^{-7} M methotrexate and the media changed every 2 days. A population of approximately 50 resistant colonies arising after three weeks in this media (CHO/E'.100) was massed and E' protein recovered as follows. The cells were collected by centrifugation, washed with phosphate buffered saline (PBS) and lysed in 2 ml of RIPA buffer (0.05M Tris-HCl pH7.5, 0.15 M NaCl, 1% Triton X-100, 1% deoxycholate and 0.1% sodium dodecyl sulfate) containing 0.5% aprotinin. The extract was preincubated for two hours with 10 mcl. of preimmune rabbit sera at 4°C, cleared twice with 50 mcl. of Pansorbin (Calbiochem), and incubated overnight with 2 mcl. of rabbit anti-LE-E' sera at 4°C. The immunoprecipitate was incubated for 30 minutes with 10 mcl. of Pansorbin, collected by centrifugation and the pellet washed twice with RIPA buffer and once with water. The immunoprecipitate is solubilized and E' separated from the rabbit antibody by ultrafiltration, electrophoresis or other conventional technique. On a commercial scale the antibody or antisera is preinsolubilized and E' eluted from the immobilized immunoabsorbent using pH 3-5 buffer.

The population of colonies arising in 1×10^{-7} M methotrexate (CHO/E'.100) produced significant amounts of two E'-encoded proteins specifically immunoprecipitated by the rabbit sera to LE-E' but not preimmune rabbit sera. Neither protein was detectable in the parent

CHO cells with the immune sera. Furthermore, immunoprecipitation of both E'-related proteins detected in CHO/E'.100 cells was readily competed by the LE-E' protein but not the LE protein, demonstrating that the reaction was specific for E' sequences.

- 5 A direct comparison shows that the two E'-related polypeptides made in CHO/E'.100 cells have electrophoretic mobilities identical with Mr 28,000 and 26,500 proteins observed in H9/HTLV-IIIB cells. In addition, the relative amounts of these two proteins in CHO/E'.100 and
- 10 H9/HTLV-IIIB cells are essentially identical. However, it is uncertain whether the 28,000 and 26,500 H9/HTLV-IIIB cell proteins are chemically identical to the E'-related recombinant polypeptides, produced herein. Pulse-labeling studies indicated that neither protein in CHO cell culture is the direct precursor of the other.
- 15 Approximately 80-90% of the Mr 28,000 and 26,500 E' protein was contained within the cytosolic fraction of transfected CHO cells. Immunoprecipitation of cell supernatants from CHO/E'.100 cultures did not reveal any secreted E' proteins.
- 20 The recombinant E'-polypeptides are sterilized by passing a solution of the polypeptides through a 0.22 micron filter in order to remove bacteria. The filtrate is formulated into a vaccine by further purifying the polypeptide, for example by gel filtration, as desired and formulating the polypeptide into a pharmaceutically-acceptable
- 25 carrier such as isotonic saline, D5W and the like. The amount of E' protein employed will be sufficient upon S.C. injection, followed by i.v. boost, to generate a detectable titer of anti-E' in the subject. This will necessarily turn on the immunocompetence of the test
- 30 subject, so the dose frequency and route of administration of antigen must be determined by the attending physician through monitoring of the serum anti-E' levels. E'-containing vaccines optionally include other predetermined AIDS-associated retroviral polypeptides such as gp41. This will be helpful in ensuring a complete potentiation of the immune response to a potential AIDS-associated retroviral infection.

The role of the E' gene protein in viral reproduction and pathogenesis remains a major unanswered question raised by these studies. Our ability to readily detect individuals who are seroreactive with LE-E' but not with whole virus antigens on immunoblots suggests that E' is not a virion component, but could also be explained if E' were lost during virus purification. Others have described unidentified proteins of Mr 25,000-28,000 in HTLV-III-infected H9 cells immunoprecipitated by AIDS-related sera (55o,55n,55p), which may correspond to E' proteins, but the same polypeptides were not detected in HTLV-III virion preparations (55o,55p). In CHO/E'.100 cells most intracellular E' was found in cytoplasmic rather than nuclear fractions, suggesting that E' does not participate in a nuclear event such as transcription initiation. These studies must be considered preliminary, however, since they may not accurately reflect the location of E' in infected cells.

Transcriptional mapping studies have suggested that E' may be an abundantly expressed protein in infected cells. Approximately 20% of the viral RNA synthesized in H9/HTLV-IIIB cells represents a family of spliced, subgenomic 1.7-1.9 kb RNAs consisting of a 289 bp leader containing sequences 5' to gag, a middle exon located upstream of env, and 1.3 kb of sequence from the 3' end of the genome containing the E' gene (55e). Either of two additional short untranslated leaders from the pol or P' regions may also be present. Depending upon alternate utilization of two splice acceptor sites (at nucleotides 5,359 and 5,558), a middle exon of either 268 bp or 69 bp is generated (55e, 55q). The 268 bp exon contains two AUG triplets not present in the 69 bp exon, one of which may serve as the initiator codon for the tat reading frame (55q). In 1.7-1.9 kb mRNAs that contain the 69 bp middle exon the predicted E' AUG initiator codon is preceded by a single AUG triplet, but we nonetheless expected it to be utilized efficiently for translation initiation since the upstream AUG triplet is flanked by unfavorable nucleotides (55r) and is followed by an in-frame termination codon well upstream of the E' AUG initiator (55s). Our ability to obtain efficient expression of E' proteins in

CHO cells with a plasmid containing the upstream AUG triplet (pSVE.E'DHFR) confirmed our expectation. Alternative usage of the two middle exons within the 4.3 kb viral mRNA class (55e), similarly determines whether the tat reading frame precedes the env gene. If differential splicing is the mechanism for generating mRNAs encoding either the E' and env proteins or the tat reading frame, it may represent a crucial regulatory event in the reproduction of the AIDS retrovirus.

- 10 E' polypeptides or antibodies thereto are employed in conventional assay procedures as are generally described above. For assay of the E' polypeptide in the body fluids of test subjects (serum, saliva, plasma, urine, etc.), a "competitive" type test kit preferably will contain labeled (enzyme, radioisotope, fluorescent group, etc.) E' polypeptide such as LE-E' or either species of E' described above, antibody capable of binding E' polypeptide and, optionally, other conventional reagents such as incubation and washing buffers. This test kit is employed in methods known per se for use with the selected label, e.g. ELISA, radioimmunoassay or fluorescence polarization.
- 15 20 Similarly, conventional "sandwich" assays also are useful in determining the presence of E' polypeptide or its antibody in test subject samples. Assays for the E' protein are unusual for AIDS diagnosis because they are the first known that are directed at determining a protein which is not present in purified preparations of the AIDS virion but which are encoded by the viral genome and apparently expressed by host cells in the course of a viral infection and/or replication in vivo. Detection of such proteins is a preferred diagnostic approach because they are believed to constitute the first indicia of viral infection, preceding the accumulation of virion antigens such as env and the generation of host antibodies directed against HTLV-III-encoded proteins.
- 25 30

It also is within the scope of this invention to assay body fluids for DNA or RNA encoding the E' protein using standard hybridization assays, e.g. Southern or Northern assays, respectively. DNA or RNA is

35

used in such assays that is merely capable of hybridizing with the Fig. 2 E'-encoding sequence; it need not encode an E' protein. Such nucleic acids are readily synthesized as oligonucleotides and then screened for the ability to hybridize to the E'-polypeptide encoding DNA set forth in Fig. 2. The diagnostic nucleic acid is labelled in conventional fashion using detectable tags such as radiophosphorus and the like.

EXAMPLE 8

10 Recombinant Synthesis of a Secreted Form of An HTLV-III Retrovirus Envelope Protein in Mammalian Cells

Figure 11a illustrates the viral genome with the location of each of the five major open reading frames of the virus. The env reading
15 frame encodes the viral envelope antigen utilized here. An envelope fragment encoding amino acid residues 61-531 was used for expression of a secreted envelope protein. This fragment was ligated to a vector which contained the components necessary for proper expression of this integrated envelope gene as well as for selection in mammalian cells.
20 "SV40 ori" contains an early promoter from the SV40 virus ("E") which is utilized to drive transcription of either the AIDS retrovirus envelope or the dihydrofolate reductase (dhfr) gene. Transcription termination and message polyadenylation are accomplished using signals derived from the 3' non-translated region of the hepatitis B virus
25 surface antigen gene ("HBV s Ag poly A signal"). Growth in E. coli is accomplished by the inclusion of the ampicillin resistance gene ("Ap R") of pBR 322 as well as the origin of replication of this plasmid. Finally, the murine dhfr gene is utilized as a selectable marker for transfection and selection in chinese hamster ovary (CHO) cells which
30 lack this gene.

A. Construction of Expression Vector

The contemplated method for assembling this vector from publicly available starting plasmids is as follows, this being a modification
35 of that method which was actually used. pE348HBV E400D22 (Simonsen et

al., 1983, "Proc. Nat. Acad. Sci.-USA" 80: 2495) is digested with HpaI and the linearized plasmid recovered. The HpaI cohesive termini are blunted with the Klenow fragment of DNA polymerase I and vector fragment 1 recovered. A blunt-ended duplex stop linker having the coding sequence TCTAGAGGATCCCCAACTAAGTAAGATCTAG is ligated to vector
5 fragment 1, the ligation mixture transfected into E. coli 294 and the vector recovered from an Amp^r colony. The underlined sequences represent stop codons in each of the three possible reading frames. The inclusion of the stop linker introduces a synthetic nine-residue
10 polypeptide at the C terminus of the env protein.

The stop-linkered vector is digested with XbaI, blunted with Klenow, digested with EcoRI and the 5706 bp vector fragment 2 recovered. This digestion serves to remove the hepatitis B surface antigen coding
15 sequence.

Clone H9c.53 described above which contained HTLV-III retroviral env cDNA is digested with HhaI, blunted with Klenow, digested with EcoRI and fragment 3, spanning nucleotides 5324 (the EcoRI site) to 7394
20 (the HhaI site at env amino acid 531), is recovered. Fragment 3 and vector fragment 2 are ligated, transfected into E. coli and vector 4 recovered from an ampicillin-resistant colony.

Vector 4 was digested with NdeI (the NdeI site corresponding to amino acid 61 of the env protein), blunted with Klenow, digested with PstI
25 and vector fragment 5 recovered. This step serves to remove the sequence located between a nucleotide located within the Amp^r gene in the 3' direction to residue 61 of the env protein, thereby removing the retroviral signal and 61 N-terminal residues of the env protein.

30 pgDtruncDHFR (EP 139,417A) is digested with PstI and PvuII and 1514 bp fragment 6 recovered containing the 3' portion of the Amp^r gene (corresponding to the sequence removed from vector 4 in the previous step), an SV40 origin, the 25 residue herpes gD protein signal and the
35 first 25 N-terminal residues of the mature gD protein. Fragments 5

and 6 are ligated, the ligation mixture transformed into E. coli 294 and pAIDSenvtrDHFR was recovered from an Amp^r colony.

The skilled artisan will appreciate that the above described vector
5 for the expression of a p41-viral protein fusion is suitably modified
for expression of other viral polypeptides than those of herpes
simplex. For example, a fusion with hepatitis surface antigen is
readily accomplished by inserting DNA encoding the mature truncated or
intact p41 protein in place of the surface antigen stop codon, or vice
10 versa, using for example pE348 HBV as a starting plasmid. Selection
of appropriate restriction sites and synthetic adaptors as required
will be within the skill of the ordinary artisan.

Other signal sequences than the herpes gD signal are used as well.
15 Suitable signals include those of secreted eukaryotic (preferably,
non-primate) polypeptides or mammalian host range viruses which are
known per se.

B. Transfection and Selection of Mammalian Cell Lines

20 On the day prior to transfection, cells from a confluent 10 cm² dish
of CHO DHFR-cells were split 1:10 into 2 10cm² dishes in F12/DMEM
(Delbecco's modified Eagles medium), 10% FBS (fetal bovine serum) +
GHT (glutamine, hypoxanthene, thymidine) + penn-strep antibiotics and
grown overnight at 37°C.

25

Preparation of Plasmid for Transfection.

75λ 10 mM Tris, 1 mM EDTA, 10 μg Plasmid DNA (omitting DNA for mock
transfection), 65λ CaCl₂ and 0.65 ml 2x hepes buffer, pH7.1 were
carefully mixed at room temperature, and thereafter poured directly on
30 the CHO cells with media. Cells and DNA were incubated at 37°C for 3
hours. The media were then aspirated and the CHO cell monolayers
shocked with 20% glycerol-PBS (2.5 ml) for 45 sec. The dishes were
flooded with 7.8 mls of F12/DMEM, aspirated and replaced with 12 ml
F12/DMEM. Cells were incubated at 37°C for 2 days and then split 1:2
35 and 1:10 in F12 media containing 7% XDZ, FBS (extensively dialyzed fetal

-70-

bovine serum), with penn-strep. The cells were fed again on day 4.

Selection and Amplification of New Cell Line.

On day 7 the mock transfected cells were all dead. The transfected CHO
5 cells split 1:2 had grown to confluence. They were split 1:3 into 3 10
cm² dishes containing F12/DMEM, 7% XDZ, 50nM MTX (Methotrexate) - 100
nM MTX and 250 nM MTX. The dishes were refed every 4 days until
confluent monolayers had adapted to MTX. These monolayers were then
labelled in ³⁵S-met and assayed by immunoprecipitation for the secreted
10 env fusion.

Cells diluted 1:10 in selection media had 50-100 colonies at day 10.
The colonies were picked and clones grown up in F12/DMEM. These clones
were screened by radioimmunoprecipitation with anti-env antisera.
Positive clones were amplified in increasing concentrations of MTX
15 (500, 1000 and 3000 nM) until maximum secretion of env fusion was
observed.

C. Radioimmunoprecipitation Analysis of Transfected CHO Cells

Confluent 10 cm dishes were labelled with 66 μ Ci/m 35S methionine for 4
hr at 37°C in RPMI 1640 medium lacking methionine. The media were then
20 collected and aprotinin was added (15 μ L). Cells were removed by
centrifugation. One ml of supernatant was added to 100 μ L of 2.5 M
NaCl, 2% Tween 80, 1- mg/ml BSA. Two μ L of high titre human anti-AIDS
antibody was added and incubated 1 hr at room temp. Protein A
Sephadex, pre-adsorbed with non-transfected CHO protein lysates, was
25 added (50 μ L) and the mixture incubated 30 minutes at 37°C. The
Sephadex beads were then washed by centrifugation 3 times in PBS, 0.2%
deoxycholate, 0.2% Tween 20. A final water wash was followed by
resuspension of the Sephadex beads in a standard SDS PAGE sample
buffer. The samples were boiled 5 minutes, centrifuged, and the
30 supernatants were separated on SDS polyacrylamide gels. The gels were
then autoradiographed using Enlighten 30.

Cells producing large amounts of envelope synthesized a ~120,000 Mr

protein which was specifically immunoprecipitable by anti HTLV-III retrovirus antibodies. This protein was found only in plasmid transfected cells. The intracellular form of the antigen was approximately 100,000 daltons in size, and it could be chased, in a pulse-chase experiment, into 120,000 dalton, secreted protein. This protein was also found to react on Western blots when incubated with anti-AIDS retrovirus antibodies.

D. Culture of Amplified Transformant CHO Cells

10 1 confluent 10 cm² dish of transformants from step B was incubated with trypsin to free the cells and transferred to a small roller bottle in 50 mls F12/DMEM 50:50 media w/o GHT, low glucose, minus glycine +7% XDZ FBS. CO₂ was introduced into the bottle for 5 sec. and capped tightly. The bottle was rolled at 37°C and observed daily until confluent (~3
15 days).

The medium was decanted, cells rinsed once in 50 mls PBS, and trypsinized for 5 min. at 37°C with 5 mls trypsin in EDTA. Cells were suspended in 5 mls of F12/DMEM 50:50 plus 7% XDZ FBS and the suspension
20 placed into 850 cm² roller bottles. This procedure is continued until the desired number of bottles is obtained. When the desired number of bottles reached about 85% confluency, they were rinsed twice in 100 mls PBS and 125 mls serum-free F12/DMEM 3:1 w/o GHT, low glucose, minus glycine added to each bottle. The bottles were incubated at 37°C for 3
25 days. On the third day the media were poured off and retained. This process was repeated twice. The ~375 ml of medium collected from each bottle was centrifuged at 2000 rpm for 5 min and Na Azide added to inhibit any contaminant microbial growth.

30 E. Purification of Recombinant HTLV III Envelope Protein

E.I. Preparation of an Affinity Column for the Isolation of Recombinant HTLV III Polypeptides.

Sera from patients diagnosed with AIDS related complex (ARC), were
35 tested for the presence of antibodies reactive with HTLV III proteins

by "Western" immunoblot analysis. Plasma from individuals exhibiting a high titers to HTLV III envelope protein were collected, and immunoglobulins were purified by affinity chromatography using Protein A-Sepharose CL-4B (Pharmacia Fine Chemicals) according to the manufacturer's directions. The purification procedure yielded material that was approximately 90% pure as judged by polyacrylamide gel electrophoresis (PAGE) visualized by silver staining. The purified immunoglobulins were then covalently linked to Sepharose 4B using CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals) according to the manufacturer's instructions.

E.II. Purification of Recombinant HTLV III Envelope Protein Fusion.

Serum free cell culture medium from the method of step D was harvested, clarified by passage through a 0.45 micron Nalgene filter, and concentrated 50-fold by ultrafiltration using an Amicon YM-10 ultrafiltration membrane. The concentrated medium was dialyzed against phosphate buffered saline (PBS), and applied to an affinity column of the type described above in E.I. After application of the concentrated cell culture medium, the column effluent was monitored spectroscopically at 280 nm and the column was washed until the effluent was free of material absorbing light at this wavelength. Proteins retained on the affinity column were eluted by treating the column with 0.1 M acetic acid, 0.5 M NaCl elution buffer. Material eluted from the column was monitored spectroscopically, and was immediately treated with 1M Tris buffer to adjust the pH to near neutrality (pH 7-8). The eluted protein was dialyzed against PBS and concentrated approximately 5 fold by ultrafiltration with the use of an Amicon YM-10 membrane. The resulting protein was analyzed by PAGE and visualized by silver staining and "Western" immunoblotting. Based on these analyses, the secreted truncated recombinant HTLV III env fusion exhibited a molecular weight of approximately 130 kd, and represented from 5-25% of the total protein eluted from the column, depending on the preparation.

E.III. Immunization of Rabbits with Affinity Purified Recombinant HTLV
III Envelope Protein Fusion.

Rabbits were immunized with 30-50 micrograms of total protein (prepared as described above) per immunization. For the primary immunization, 5 30-50 micrograms of protein in 1 ml of PBS was emulsified with an equal volume of complete Freund's adjuvant and injected as follows: 0.5 ml in each rear footpad, 0.2 ml injected at 5 intradermal sites along the back. The rabbits were then boosted 14-21 days after the primary immunization. The antigen emulsion for the booster immunizations was 10 prepared the same way as that for the primary immunization with the exception that incomplete Freund's adjuvant replaced complete Freund's adjuvant. For the booster immunizations each rabbit received a total of 2 ml of antigen emulsion distributed as follows: 0.5 ml intramuscularly in each thigh, and 1 ml distributed among 5 intradermal 15 sites along the back (0.2 ml per site). Animals were bled via the ear vein or cardiac puncture 10 to 14 days after each boost. Antibodies elicited against the recombinant HTLV III envelope protein were identified by "Western" immunoblot analysis using recombinant HTLV III 130K protein as the antigen. In the first experiment, one of two 20 rabbits formed antibodies reactive with the recombinant HTLV III protein after one booster immunization. The second rabbit required additional booster immunizations to elicit the formation of anti-rHTLV III antibodies.

25 F. Neutralization of HTLV III Retrovirus Infection

Various sera from either control rabbits, rabbits inoculated with the recombinant envelope antigen, normal control patients, or patients with known retrovirus neutralizing antibodies were heat inactivated at 56°C for 30 minutes. The serum samples were then mixed 1:1 with virus in 30 wells containing RPMI 1640, 20% fetal calf serum, penn-strep, and 2 µg/ml polybrene. The wells were incubated 1 hour at 4°C, after which the plates were returned to room temperature for 15 minutes. 1×10^6 H9 human T cells were then added to each well, and the wells were incubated in CO₂ incubator. The cultures were split 1:1 on day 4. 35 Cells were assayed for reverse transcriptase and immunofluorescence on

day 7 or 8. Immunofluorescence assays were done on fixed cells using human antisera known to contain AIDS retrovirus antibodies. The percentage of cells which were fluorescent was a measure of the percentage of cells which were infected by the virus. Reverse transcriptase assays of cell supernatants were done as described. The reduction in reverse transcriptase levels was indicative of virus neutralization. When either control human or rabbit sera were mixed with the virus, approximately 70-80% of the cells were fluorescent. Reverse transcriptase assays gave approximately 1.6×10^6 cpm incorporated. When serum from a rabbit vaccinated with recombinant envelope antigen was analyzed, the percentage of cells fluorescing was 35% and the reverse transcriptase levels were 285,000 cpm. Human serum known to neutralize the virus gave 0% fluorescence and 10,000 cpm reverse transcriptase. These results indicate neutralization with the antiserum from rabbits vaccinated with the recombinant antigen.

Although the foregoing refers to particular preferred embodiments, it will be understood that the present invention is not so limited. It will occur to those ordinarily skilled in the art that various modifications may be made to the disclosed embodiments and that such modifications are intended to be within the scope of the present invention.

The references cited herein or grouped in the following bibliography and respectively cited parenthetically by number in the foregoing text, are hereby incorporated by reference.

BIBLIOGRAPHY

1. Entage et al., Nature 283, 171 (1980); Davis, et al., Proc. Natl. Acad. Sci. (USA) 78, 5376 (1981); Weiland, et al., Nature 292, 851 (1981).
2. Kupper, et al., Nature 289, 555 (1981); Kleid et al., Science 214, 1125 (1981).
3. Charnay et al., Nucleic Acids Research 7, 335 (1979); Valenzuela et al., Nature 298, 247 (1982).
4. Rose et al., PNAS 78, 6670 (1981).
5. Yelverton et al., Science 219, 614 (1983).
6. Burrell, C. et al., Nature 291, 503-506 (1981); Mackay et al., PNAS 78, 4510-4514 (1981).
7. Fauci, A. et al., Annals of Internal Medicine 100, 92 (1984).
8. Marx, J.L., Science 224, 475 (1984).
9. Groopman, J.E., Nature 308, 769 (1984).
10. Wilson, T., Biotechnology p. 682 August (1984).
11. Gallo, R.C. et al., Science 224, 500 (1984).
12. Popovic, M. et al., Science 224, 497 (1984).
13. Barre-Sinouss, F. et al., Science 220, 868 (1983).
14. Vilmer, F. et al., Lancet 753, (1984).
15. Chemical and Engineering News, 14 Sept. (1984).
16. Sarngadharen, M.G. et al., Science 224, 506 (1984).
17. Kitchen, L.W. et al., Nature 312, 367 (1984).
18. Bolivar et al., Gene 2, 95 (1977).
19. Chang et al., Nature 275, 615 (1978).
20. Itakura et al., Science 198, 1056 (1977).
21. Goeddel et al., Nature 281, 544 (1979).
22. Goeddel et al., Nucleic Acids Res. 8, 4057 (1980).

23. European Patent Appln. Publ. No. 036776.
24. Siebenlist et al., Cell 20, 269 (1980).
25. Stinchcomb et al., Nature 282, 39 (1979).
26. Kingsman et al. Gene 7, 141 (1979).
27. Tschemper et al., Gene 10, 157 (1980).
28. Jones, Genetics 85, 12 (1977).
29. Hitzeman et al., J. Biol. Chem. 255, 2073 (1980).
30. Hess et al. J. Adv. Enzyme Reg. 7, 149 (1968).
31. Holland et al. Biochemistry 17, 4900 (1978).
32. T. Maniatis et al., Molecular Cloning (Cold Spring Harbor Press. N.Y. 1982)
33. Mandel et al. J. Mol. Biol. 53, 154 (1970).
34. R. Lawn et al. Nucleic Acids Res. 9, 6103-6114 (1981).
35. Crea et al., Proc. Natl. Acad. Sci. USA 75(12), 5765 (1978).
36. Deleted
37. Wood, N.I., et al., Nature 312, 330 (1984).
38. Gray, P.W. et al., Nature 295, 503 (1982).
39. Messing, R. et al., Nucleic Acid Res. 9, 309-321 (1981).
40. Laemelli, U. Nature 227, 680-685 (1970).
41. Hunkapillar, M. et al. in Methods in Enzymology, 91, 227-231 (eds. Hirs, C. and Timasheff, S.) (Academic Press, N.Y., 1983).
42. Hewick, R.M. et al., J. Biol. Chem. 256:7990 (1981).
43. Vieira, J. and Messing, J. GEM, 19, 259-268, (1982).
44. McGrath et al., Nature 295, 423 (1982).
45. DeBoer, H. et al. in Promoters, Structure and Function (Praeger Scientific, 1982).
46. Liu, C. et al., Nature 309, 82 (1984).
47. Kirid, D., et al., Science, 214, 1125-1129 (1981).

- 48. Gazdar, A.F. et al., Blood 55, 409 (1980).
- 49. Gallo, R. et al., PNAS, 77, 7415 (1980).
- 50. No reference
- 51. No reference
- 52. No reference
- 53. Mitsua, H. et al., Science 226, 172 (1984).
- 54. DeCiccio, J. Nat. Cancer Institute 61, 1187 (1978); Verma, B.B.A. 473 (1974).
- 55a. Kleid, D. et al., Science 214, 1125-1129 (1981).
- 55b. Miozzaro, G. and Yanofsky, C., J. Bact. 133, 1457-1466 (1978).
- 55c. Goeddel, D. et al., Proc. Natl. Acad. Sci. 76, 106-110 (1979).
- 55d. Towbin, H., Staehelin, T. and Gordon, T., Proc. Natl. Acad. Sci. 76, 4350-4353 (1979).
- 55e. Muesing, M. et al., Nature 313, 450-458 (1985).
- 55f. Urlaub, G. and Chasin, L., Proc. Natl. Acad. Sci. 77, 4216-4220 (1980).
- 55g. Graham, F. and van der Eb, A., Virology 52, 456-467 (1977).
- 55h. Wigler, M. et al., Proc. Natl. Acad. Sci. 76, 1373-1376 (1979).
- 55i. Frost, E. and Williams J., Virology 91, 39-50 (1978).
- 55j. Ringold, G., Dieckmann, B. and Lee, F., J. Mol. Appl. Genet. 1, 165-175 (1981).
- 55k. Kaufman, R. and Sharp, P., J. Mol. Biol. 159, 601-621 (1982).
- 55l. Safai, B. et al., Lancet I:1438-1440 (1984).
- 55m. Salahuddin, S. et al., Lancet II:1418-1420 (1984).
- 55n. Barin, F. et al., Science 228, 1094-1096 (1985).
- 55o. Veronese, F. et al., Science 229, 1402-1405 (1985).
- 55p. Robey, W. et al., Science 228, 593-595 (1985).
- 55q. Arya, S., Chan, G., Josephs, S. and Wong-Staal, F., Science 229, 69-73 (1985).

- 55r. Kozak, M., Nucleic Acids Res. 9, 5233-5253 (1981).
- 55s. Liu, C., Simonsen, C. and Levinson, A., Nature 309, 82-85 (1984).
- 56. Rose, D. et al., Proc. Nat. Acad. Sci. USA, 78, 6670 (1981).
- 57. Yelverton, E. et al., Science 219, 614 (1983).
- 58. Voller, A. et al., Manual of Clinical Immunology 2d (1980), American Society for Microbiology, pp. 359-371.

CLAIMS

1. A composition comprising a predetermined polypeptide encoded by the genome of an AIDS associated retrovirus which
5 composition is essentially free of other naturally occurring AIDS associated polypeptides or human proteins from cells for which the AIDS-associated retrovirus is naturally infective, said predetermined polypeptide sequence having at least one antigenic determinant capable of specifically
10 binding complementary antibody.
2. The composition of claim 1 wherein said predetermined polypeptide sequence is a sequence encoded by the gag region of the genome of an AIDS associated retrovirus.
15
3. The composition of claim 1 wherein said predetermined polypeptide sequence is derived from a sequence encoded by the env region of the genome of an AIDS associated retrovirus.
20
4. The composition of claim 1 wherein said predetermined polypeptide sequence is from gp-41, gp-65 or gp-120.
5. The composition of claim 1 wherein a normally glycosylated predetermined polypeptide sequence of an AIDS
25 associated retrovirus is not glycosylated.
6. The composition of any one of the preceding claims wherein said predetermined polypeptide sequence contains
30 methionine or formylmethionine at the N-terminus.
7. The composition of any one of the preceding claims wherein said predetermined polypeptide sequence is a fusion of the AIDS associated retroviral polypeptide with a viral
35 or bacterial polypeptide.

8. A process of comprising isolating a predetermined polypeptide sequence of an AIDS associated retrovirus from a prokaryotic or mammalian recombinant expression host containing DNA sequence encoding said predetermined polypeptide sequence, wherein the isolated predetermined polypeptide sequence contains at least one antigenic determinant capable of specifically binding complementary antibody.
9. The polypeptide product made by the process of claim 8.
10. A composition comprising a predetermined fragment of a predetermined polypeptide sequence of an AIDS associated retrovirus, said fragment containing at least one antigenic determinant capable of specifically binding complementary antibody.
11. The composition of claim 10 essentially free of other AIDS associated viral polypeptides.
12. The composition of claim 10 or claim 11 wherein said polypeptide fragment is an N-terminal sequence of a predetermined polypeptide sequence of an AIDS associated retrovirus.
13. The composition of claim 10 or claim 11 wherein said polypeptide fragment is a C-terminal sequence of a predetermined polypeptide sequence of an AIDS associated retrovirus.
14. The composition of claim 13 wherein said polypeptide fragment is a fragment of the AIDS associated gp-120 env protein.
15. The composition of claim 10 wherein said polypeptide fragment is from a sequence encoded by the gag region of the

genome of an AIDS associated retrovirus.

16. The composition of claim 10 wherein said polypeptide fragment is from p-24.

5

17. The composition of claim 10 wherein said polypeptide fragment is from a sequence encoded by the env region of the genome of an AIDS associated retrovirus.

10 18. The composition of claim 10 wherein said polypeptide fragment is from gp-41, gp-65 or gp-120.

15 19. A fusion polypeptide comprising (a) a first predetermined polypeptide sequence or fragment thereof, of an AIDS associated retrovirus having at least one antigenic determinant capable of specifically binding complementary antibody and (b) a second polypeptide sequence which is not immunologically cross-reactive with antibodies normally present in a biologically derived sample which is to be
20 assayed for the presence of said complementary antibody.

20. The fusion polypeptide of claim 19 wherein said predetermined polypeptide sequence is as defined in any one of claims 15 to 18.

25

21. The fusion polypeptide of claim 19 or claim 20 wherein the first and second polypeptide sequences are fused.

22. The fusion polypeptide of claim 21 wherein said fusion
30 is by a peptide bond.

23. The fusion polypeptide of claim 21 or claim 22 wherein the amino terminus of the first polypeptide sequence is fused to the carboxyl terminus of the second polypeptide
35 sequence.

24. A composition comprising a variant polypeptide sequence of an AIDS associated retrovirus containing at least one antigenic determinant capable of specifically binding complementary antibody to an AIDS associated
5 retrovirus.
25. The composition of claim 24 wherein said variant polypeptide sequence is an insertion, deletion or substitution of an amino acid residue of a polypeptide sequence of an
10 AIDS associated retrovirus.
26. The composition of claim 24 or claim 25 wherein said variant polypeptide sequence is a labelled or bound derivative of a polypeptide sequence of an AIDS associated
15 retrovirus.
27. A diagnostic test kit comprising the composition of any one of claims 1 to 7 and 9 to 26.
- 20 28. The composition of any one of claims 1 to 7 and 9 to 26 which is immobilized on a solid phase.
29. The composition of any one of claims 1 to 7, 9 to 26 and 28 which is labelled with a detectable marker.
25
30. A vaccine comprising the composition of any one of claims 1 to 7 and 9 to 25, wherein said polypeptide is capable of inducing the production of neutralizing antibodies which confer resistance to infection by AIDS
30 associated retrovirus.
31. The vaccine of claim 30 wherein the AIDS-associated polypeptide is a fusion with a second polypeptide and said second polypeptide does not normally induce antibodies which
35

cross-react with proteins which are naturally occurring in the subject such vaccine is directed to.

5 32. The vaccine of claim 31 wherein the second polypeptide is a trpLE fusion.

33. The vaccine of any one of claims 30 to 32 including a pharmaceutically acceptable vehicle.

10 34. A DNA sequence encoding the composition of any one of claims 19 to 25.

15 35. A DNA sequence encoding a fusion polypeptide of any one of claims 1 to 7 and 18 to 25 wherein said DNA sequence is essentially free of DNA complementary to the naturally occurring flanking RNA sequences.

20 36. A replicable prokaryotic or mammalian cell expression vector containing the DNA sequence of claims 34 or 35 which is capable of transforming a host cell to expression of an AIDS associated polypeptide.

25 37. A prokaryotic or mammalian cell culture containing the expression vector of claim 36.

38. A method for the detection of antibody contained in a test sample comprising:

30 a) contacting said sample with the composition of claims 28 or 29 to bind the diagnostic product with complementary antibody in said sample; and

b) detecting the amount bound or unbound detectable marker.

35 39. The method of claim 38 wherein the test sample is urine, saliva or a blood fluid.

40. A reverse transcriptase of an AIDS associated retrovirus which is essentially free of other AIDS associated retrovirus proteins.

5 41. A cell-free DNA sequence encoding the reverse transcriptase of claim 40.

42. A replicable expression vector containing the DNA sequence of claim 41, said vector being capable of expressing reverse transcriptase when contained within a transformed cell.

43. A eukaryotic cell culture containing the expression vector of claim 42.

15 44. The culture of claim 43 wherein the cells are those of a mammalian cell line.

45. An assay for identifying compounds which inhibit reverse transcriptase of an AIDS associated retrovirus comprising:

a) reacting the reverse transcriptase of an AIDS associated retrovirus with a compound which is suspected to be capable of inhibiting said reverse transcriptase; and

25 b) measuring the level of reverse transcriptase activity of the reaction mixture of step a).

46. The assay of claim 45 wherein the reverse transcriptase is present in a transformed recombinant mammalian host cell.

47. A composition comprising an AIDS-associated retroviral E' polypeptide, which composition is free of proteins from AIDS-associated retrovirus infected cells that are not

encoded by the AIDS-associated retrovirus and which is free of infectious AIDS-associated retroviral virions.

48. The composition of claim 47 wherein the E' polypeptide
5 has a relative molecular weight on SDS PAGE of about 28,000 or about 26,500.

49. The composition of claim 47 wherein the E' polypeptide has the amino acid sequence of the E' polypeptide of Fig. 2
10 or its naturally-occurring alleles.

50. A composition comprising an E' polypeptide which is other than the E' polypeptide of Fig. 2 or its naturally-occurring alleles but which is immunologically cross-
15 reactive with the E' polypeptide of Fig. 2 or its naturally-occurring alleles.

51. The composition of any one of claims 47 to 49 which is a vaccine.
20

52. The composition of any one of claims 47 to 49 which is sterile, comprises a pharmaceutically-acceptable carrier, and contains the E' polypeptide in an amount sufficient upon administration to an animal to elicit the formation of anti-
25 bodies to the E' polypeptide.

53. The composition of any one of claims 47 to 49, 51 and 52 which further contains known amounts of other predetermined AIDS-associated retroviral polypeptides.
30

54. A diagnostic test kit for AIDS-associated retroviral infection comprising the composition of any one of claims 47 to 50.

35 55. The composition of any one of claims 47 to 50 wherein

the E' polypeptide is labelled with a detectable marker.

56. The composition of claim 55 wherein the detectable marker is an enzyme, radioisotope or fluorescent substituent.

5

57. The composition of claim 54 wherein the test kit comprises an immobilized antibody capable of binding the E' polypeptide, said antibody being in a container separate from the E' polypeptide.

10

58. An antibody capable of binding an AIDS-associated retroviral E' polypeptide which is free of antibody capable of binding any other AIDS-associated retroviral-encoded polypeptide and which is free of bound E' polypeptide.

15

59. The antibody of claim 58 which is immobilized.

60. The antibody of claim 59 which is immobilized by adsorption to a polyolefin or to antibody capable of binding the constant region of the antibody of claim 59.

20

61. A method for the early determination of the presence of an AIDS-associated retroviral infection in a test subject comprising:

25

- (a) obtaining a sample from the test subject;
- (b) detecting the presence in the test sample of a polypeptide encoded by the RNA of the AIDS-associated retrovirus which is other than a polypeptide component of the purified AIDS-associated retrovirus virion.

30

62. The method of claim 61 wherein the detected polypeptide is an E' polypeptide.

63. The method of claim 61 or claim 62 further comprising additionally detecting the presence of a predetermined poly-

35

peptide component of the AIDS-associated retrovirus virion.

64. Nucleic acid encoding an AIDS-associated retroviral E' polypeptide free of flanking proviral sequences encoding
5 other complete AIDS-associated retroviral polypeptides.

65. A replicable vector comprising the nucleic acid of claim 64.

10 66. The nucleic acid of claim 64 which is labelled with a detectable substituent.

CLAIMS

1. A process which comprises the preparation of a composition comprising a predetermined polypeptide encoded by the genome of an AIDS associated retrovirus which composition is essentially free of other naturally occurring AIDS associated polypeptides or human proteins from cells for which the AIDS-associated retrovirus is naturally infective, said predetermined polypeptide sequence having at least one antigenic determinant capable of specifically binding complementary antibody.
2. The process of claim 1 wherein said predetermined polypeptide sequence is a sequence encoded by the gag region of the genome of an AIDS associated retrovirus.
3. The process of claim 1 wherein said predetermined polypeptide sequence is derived from a sequence encoded by the env region of the genome of an AIDS associated retrovirus.
4. The process of claim 1 wherein said predetermined polypeptide sequence is from gp-41, gp-65 or gp-120.
5. The process of claim 1 wherein a normally glycosylated predetermined polypeptide sequence of an AIDS associated retrovirus is not glycosylated.
6. The process of any one of the preceding claims wherein said predetermined polypeptide sequence contains methionine or formylmethionine at the N-terminus.
7. The process of any one of the preceding claims wherein said predetermined polypeptide sequence is a fusion of the AIDS associated retroviral polypeptide with a viral or

bacterial polypeptide.

8. A process of comprising isolating a predetermined polypeptide sequence of an AIDS associated retrovirus from a prokaryotic or mammalian recombinant expression host containing DNA sequence encoding said predetermined polypeptide sequence, wherein the isolated predetermined polypeptide sequence contains at least one antigenic determinant capable of specifically binding complementary antibody.
9. The polypeptide product made by the process of claim 8.
10. A process which comprises the preparation of a composition comprising a predetermined fragment of a predetermined polypeptide sequence of an AIDS associated retrovirus, said fragment containing at least one antigenic determinant capable of specifically binding complementary antibody.
11. The process of claim 10 wherein the composition is essentially free of other AIDS associated viral polypeptides.
12. The process of claim 10 or claim 11 wherein said polypeptide fragment is an N-terminal sequence of a predetermined polypeptide sequence of an AIDS associated retrovirus.
13. The process of claim 10 or claim 11 wherein said polypeptide fragment is a C-terminal sequence of a predetermined polypeptide sequence of an AIDS associated retrovirus.
14. The process of claim 13 wherein said polypeptide fragment is a fragment of the AIDS associated gp-120 env protein.
15. The process of claim 10 wherein said polypeptide fragment is from a sequence encoded by the gag region of the genome of an AIDS associated retrovirus.

16. The process of claim 10 wherein said polypeptide fragment is from p-24.

5 17. The process of claim 10 wherein said polypeptide fragment is from a sequence encoded by the env region of the genome of an AIDS associated retrovirus.

18. The process of claim 10 wherein said polypeptide fragment is from gp-41, gp-65 or gp-120.

10

19. A process which comprises the preparation of a fusion polypeptide comprising (a) a first predetermined polypeptide sequence or fragment thereof, of an AIDS associated retrovirus having at least one antigenic determinant capable of
15 specifically binding complementary antibody and (b) a second polypeptide sequence which is not immunologically cross-reactive with antibodies normally present in a biologically derived sample which is to be assayed for the presence of said complementary antibody.

20

20. The process of claim 19 wherein said predetermined polypeptide sequence is as defined in any one of claims 15 to 18.

25 21. The process of claim 19 or claim 20 wherein the first and second polypeptide sequences are fused.

22. The process of claim 21 wherein said fusion is by a peptide bond.

30

23. The process of claim 21 or claim 22 wherein the amino terminus of the first polypeptide sequence is fused to the carboxyl terminus of the second polypeptide sequence.

24. A process which comprises the preparation of a composition comprising a variant polypeptide sequence of an AIDS associated retrovirus containing at least one antigenic determinant capable of specifically binding complementary
5 antibody to an AIDS associated retrovirus.

25. The process of claim 24 wherein said variant polypeptide sequence is an insertion, deletion or substitution of an amino acid residue of a polypeptide sequence of an AIDS
10 associated retrovirus.

26. The process of claim 24 or claim 25 wherein said variant polypeptide sequence is a labelled or bound derivative of a polypeptide sequence of an AIDS associated
15 retrovirus.

27. A diagnostic test kit comprising the composition as defined in any one of claims 1 to 7 and 9 to 26.

20 28. A device comprising a composition as defined in any one of claims 1 to 7 and 9 to 26 immobilized on a solid phase.

29. A device comprising a composition as defined in any
25 one of claims 1 to 7, 9 to 26 and 28 labelled with a detectable marker.

30. A process which comprises the preparation of a vaccine comprising the composition as defined in any one of claims 1
30 to 7 and 9 to 25, wherein said polypeptide is capable of inducing the production of neutralizing antibodies which confer resistance to infection by AIDS associated retrovirus.

31. The process of claim 30 wherein the AIDS-associated
35 polypeptide is a fusion with a second polypeptide and said

second polypeptide does not normally induce antibodies which cross-react with proteins which are naturally occurring in the subject such vaccine is directed to.

5 32. The process of claim 31 wherein the second polypeptide is a trpLE fusion.

33. The process of any one of claims 30 to 32 wherein the vaccine includes a pharmaceutically acceptable vehicle.

10

34. A process which comprises the preparation of a DNA sequence encoding the composition as defined in any one of claims 19 to 25.

15 35. A process which comprises the preparation of a DNA sequence encoding a fusion polypeptide as defined in any one of claims 1 to 7 and 18 to 25 wherein said DNA sequence is essentially free of DNA complementary to the naturally occurring flanking RNA sequences.

20

36. A process which comprises the preparation of a replicable prokaryotic or mammalian cell expression vector containing the DNA sequence as defined in claim 34 or claim 35 which is capable of transforming a host cell to expression
25 of an AIDS associated polypeptide.

37. A process which comprises transforming a prokaryotic or mammalian cell culture with an expression vector as defined in claim 36.

30

38. A method for the detection of antibody contained in a test sample comprising:

a) contacting said sample with the composition as defined in claims 28 or 29 to bind the diagnostic product
35 with complementary antibody in said sample; and

b) detecting the amount bound or unbound detectable marker.

39. The method of claim 38 wherein the test sample is
5 urine, saliva or a blood fluid.

40. A process which comprises the preparation of a reverse transcriptase of an AIDS associated retrovirus which is essentially free of other AIDS associated retrovirus
10 proteins.

41. A process which comprises the preparation of a cell-free DNA sequence encoding the reverse transcriptase as defined in claim 40.
15

42. A process which comprises the preparation of a replicable expression vector containing the DNA sequence as defined in claim 41, said vector being capable of expressing reverse transcriptase when contained within a transformed
20 cell.

43. A process which comprises transforming a eukaryotic cell culture with an expression vector as defined in claim 42.
25

44. The process of claim 43 wherein the cells are those of a mammalian cell line.

45. An assay for identifying compounds which inhibit
30 reverse transcriptase of an AIDS associated retrovirus comprising:

a) reacting the reverse transcriptase of an AIDS associated retrovirus with a compound which is suspected to be capable of inhibiting said reverse transcriptase; and
35 b) measuring the level of reverse transcriptase

activity of the reaction mixture of step a).

46. The assay of claim 45 wherein the reverse transcrip-
tase is present in a transformed recombinant mammalian host
5 cell.

47. A process which comprises the preparation of a compos-
ition comprising an AIDS-associated retroviral E' polypep-
tide, which composition is free of proteins from AIDS-
10 associated retrovirus infected cells that are not encoded by
the AIDS-associated retrovirus and which is free of infec-
tious AIDS-associated retroviral virions.

48. The process of claim 47 wherein the E' polypeptide has
15 a relative molecular weight on SDS PAGE of about 28,000 or
about 26,500.

49. The process of claim 47 wherein the E' polypeptide has
the amino acid sequence of the E' polypeptide of Fig. 2 or
20 its naturally-occurring alleles.

50. A process comprising an E' polypeptide which is other
than the E' polypeptide of Fig. 2 or its naturally-occurring
alleles but which is immunologically cross-reactive with the
25 E' polypeptide of Fig. 2 or its naturally-occurring alleles.

51. The process of any one of claims 47 to 49 wherein the
composition is a vaccine.

30 52. The process of any one of claims 47 to 49 which pro-
duces a composition which is sterile, comprises a
pharmaceutically-acceptable carrier, and contains the E'
polypeptide in an amount sufficient upon administration to
an animal to elicit the formation of antibodies to the E'
35 polypeptide.

53. The process of any one of claims 47 to 49, 51 and 52 wherein the composition further contains known amounts of other predetermined AIDS-associated retroviral polypeptides.
- 5 54. A diagnostic test kit for AIDS-associated retroviral infection comprising the composition as defined in any one of claims 47 to 50.
55. a device comprising a composition as defined in any
10 one of claims 47 to 50 wherein the E' polypeptide is labeled with a detectable marker.
56. The device of claim 55 wherein the detectable marker is an enzyme, radioisotope or fluorescent substituent.
- 15 57. The test kit of claim 54 which comprises an immobilized antibody capable of binding the E' polypeptide, said antibody being in a container separate from the E' polypeptide.
- 20 58. A process which comprises the preparation of an antibody capable of binding an AIDS-associated retroviral E' polypeptide which is free of antibody capable of binding any other AIDS-associated retroviral-encoded polypeptide and
25 which is free of bound E' polypeptide.
59. A device comprising an antibody as defined in claim 58 which is immobilized.
- 30 60. The device of claim 59 wherein the antibody is immobilized by adsorption to a polyolefin or to antibody capable of binding the constant region of the antibody of claim 59.

61. A method for the early determination of the presence of an AIDS-associated retroviral infection in a test subject comprising detecting the presence in a sample from the test subject of a polypeptide encoded by the RNA of the AIDS-
5 associated retrovirus which is other than a polypeptide component of the purified AIDS-associated retrovirus virion.

62. The method of claim 61 wherein the detected polypeptide is an E' polypeptide.

10

63. The method of claim 61 or claim 62 further comprising additionally detecting the presence of a predetermined polypeptide component of the AIDS-associated retrovirus virion.

15 64. A process which comprises the preparation of nucleic acid encoding an AIDS-associated retroviral E' polypeptide free of flanking proviral sequences encoding other complete AIDS-associated retroviral polypeptides.

20 65. A process which comprises the preparation of a replicable vector comprising the nucleic acid as defined in claim 64.

66. A device which comprises the nucleic acid as defined
25 in claim 64 labelled with a detectable substituent.

1/25

51

01

5

Fig. 2a.

1 GGTCTCTCTGGTTAGACCAGATCTAGGCTGGGAGCTCTCTGGCTAACTAGGGAACCACTGCTTAAGCCTCAATAAGCCTTGCCCTTGAGTGTCAAGT^RU5
 101 AGTGTGTGCCCGTCTGTGTGACTCTGGTAAC TAGAGATCCCTCAGACCCCTTTTAGTCAAGTGTGGAAATCTCTAGCAGTGGCGCCCGAACAAGGACI^{U6}
 201 IGAAAGCGAAAGGGAAACAGAGGAGCTCTCTCGACGCAGGACTCGGCTTGTGAAGCGCGCACGGCAAGAGGGCGGCGGACTGGTGAGTACGCC^{sd}
 301 AAAAAATTTTGACTAGCGGAGGCTAGAAAGGAGAGAGATGGGTGGCAGAGCGTCAATTAAGCGGGGAGAAATAGATCGATGGGAAAAATTCGGTTAAG^{IF}
 401 GCCAGGGGGAAGAAAAATATAAATAAACAATAGTATGGCAAGCAGGAGCTAGAACGATTCGCAGTTAATCCTGGCCTGTTAGAAACATCAGAA^{gag}
 501 GGCCTAGACAATACTGGGACAGCTACAACCATCCCTTCAGACAGGATCAGAAAGACTTAGATCATTAATAATACAGTAGCAACCCCTCTATTGTGTG^{Met}
 601 ATCAAGGATAGAGATAAAGACACCAAGGAGCTTTAGACAAGATAGAGGAAGAGCAAAACAAAGTAAGAAAAAGCAGCAAGCAGCAGCTGACAC^{p24gag}
 701 AGGACACAGCAGTCAGGTCAGCCAAAATTACCTATAGTGCAGAACATCCAGGGGCAATGGTACATCAGGCCATATCACCTAGAACTTTAAATGCATGG
 801 GTAAAAGTAGTAGAAGAGAAGGCTTCAGGCCAGAAAGTGTACCATGTTTTCAGCATTTATCAGAAAGGAGCCACCCCAAGATTTAAACACCATGCTAA^{U6}
 901 ACACAGTGGGGGACATCAAGCAGCCATGCAAAATGTTAAAGAGAGACCATCAATGAGGAAGCTGCAGAAATGGGATAGAGTGCAATCCAGTGCATGCAGGGCC^{PBS}

Fig. 2b.

```

1001 ILEALAPROGLYGLNMetARGGLUPROARGGLYSERASPILEALAGLYTHRThrSERThrLEUGLLeuGLNLeuILEGLYTrpMetThrASNASNProPRO
TATTGCAACAGGCCAGATGAGAGAACCAGGGGAAGTGAArgCATAGCAGGAACArgTACTAGTACCCArgTCAGGAACAAATAGGAArgTGGATGACAAATATATCCACCTArg

1101 ILEPROVALGLYGLUILEThrLYSARGTrpILEILEUGLYLEUASNLYSILEVALARGMetTYRSERProTHRSerILELEUASPLeuILEARGGLNGLYPro
ATCCCAGTAGGAGAAATTTATAAAGATGGATAATCTCTGGGATTAATAAATAGTAAGAATGTATAGCCCTACCAGCATTCArgTGGACATAAGACAAGGACArg

1201 LYSGLUPROPHEARGASPIYRVALASPARGPHEArgTYRLeuYSThrLEUARGALAGLUNALASERGLNGLUVALLYSASNTrpMetThrGLUThrLEULEU
CAAAAGAACCCTTTAGAGACTATGTAGACCGGTTCATAAAACTCTAAGAGCCGAGCAAGCTTCACAGGAGGTAAAAAATArgTGGATGACAGAAACCTTGTArg

1301 VALGLNASNALASNPROASPCYSLeuYSThrILELEULYSALALEUGLYPROALAALATHRLeuLEUGLUGLUMETMetTHRAlaCYSGLNGLYVALGLYGLY
GGTCCAAAATGCGAACCCAGATTGTAAGACTATTTAAAGCATTTGGGACCAGCAGCTACACTAGAAAGAAATGATGACAGCATGTArgCAGGGAGTGGGAGGAArg

1401 PROGLYHISLYSALAARGVALLEUALAGLUALAMETSERGLNVALThrASNThrALAThrILEMetMetLeuGLNARGGLYASNPheARGASNGLNARGLYMet
CCCGGCCATAAGGCAAGAGTTTGGCTGAAGCAATGAGCCAGTAAACAAATACAGCTACCAATAATGATGACAGAGAGGCAATTTTAGGAACCAAAGAAAGAArg

1501 VALLYSCYSPHEASNCysGLYLYSGLUGLYHISThrALAAARGASNCysARGALAPROARGLYSLeuGLYCYSTRPLCysGLYLYSGLUGLYHISGLN
TGGTTAAGTGTTTCAATTGTGGCAAGAAGAGGGCACACAGCCAGAAATTTGAGGGCCCTTAGGAAAAAGGGCTGTArgTGGAAATGTGGAAAGGAAGGACACCAArg

1601 METLYASPCYSThrGLUARGGLNALAASNPheLEUGLYLYSILECysLEUPROThrARGGLUGLYGLNGLYILEPhePHEPheARGALAAArgSPGLNSerGLN
AATGAAAGAATTGTACTGAGAGACAGGCTAATTTTArgAGGAAGATCTGCCArgTTCTACAAAGGGAAGGCCAGGGAAATTTCTArgTCAGAGCAGGACCCAGAGCCAAArg

1701 SERPROThrILESerSERSerGLUGLThrARGALAAASNSerPROThrARGARGGLULEUGLNValTRPGLYARGASPasASNSerPROSerGLUALAGLY
GLNProHISHisPHEPheARGALAAArgSPGLNSerGLNProHISGLNLYSARGALASERGLYLEUGLYAM*
CAGCCCCACCATTTCTTCAGAGCAGACCAGAGCCCAACAGCCCCCACCAGAGAGAGCTTCAGGTCTGGGGTArgAGAGACAACAACArgCTCCCTCAGAAAGCAGGAArg

1801 ALAASPARGGLNGLYThrVALSERPheASNPhePROGLNILEThrLEUTrpGLNARGProLEUVALThrILELYSILEGLYGLNLeuLYSGLUALALEU
GCCGATAGACAAGGAACArgTATCCTTTAACTTCCCTCAGATCACTCTTTGGCAACGACCCCTCGTCACTACCAATAAAGATAGGGGGGCAACTAAAGGAAGCTCArg

1901 LEUASPThrGLYALAASPASPThrVALLEUGLUMETSerLEUPROGLYARGTrpLYSProLYSMetILEGLYGLYILEGLYGLYPheILELYSVAL
TATTAGATACAGGAGGAGATGATACAGTATArgTAGAAGAAATGAGTTTGCAGGAAGATGGAAACCAAAAATGATAGGGGGAAATGGAGGTTTATCAAAGTArg

```

Fig. 2c.

ARGGLNTRYASPGLNILELEUILEGUILECYSGLYHISLYSALALEGLYTHRVALLEUVALGLYPROTHRPROVALASNILEILEGLYARGASNLEU
 2001 AAGACAGTATGATCAAAATCTCATAGAAATCTGTGGACAATAAGCTATAGGTACAGTATAGTAGGACCTACACCTGTCAACATAATTTGGGAAGAAATCTG
 LEUTHRGLNILEGLYCYSYTHRLEUASNPHLEPROILESERPROILEGLUTHRVALPROVALLYSLEULYSYSPROGLYMETASPGLYPROLYSVALLYSGLNTRP
 2101 TTGACTCAGATTGGTTGCACTTTAAATTTTCCCATTAGCCCTATTGAGACTGTACCAGTAAAATTAAGGCCAGGAATGGATGGCCCCAAAAGTTAAACAAI
 PROLEUTHRGLUGLULYSILELYSALALEUVALGLUILECYSTHRGLUMETGLULYSGLUGLYLYSILESERLYSILEGLYPROGLUASNPROTYRASN
 2201 GGCCATTGACAGAGAAGAAAAATAAAGCAATTAGTAGAAAATTTGTACAGAAATGGAAAAAGGAAGGGAAAAATTTCAAAAAATTTGGGCCCTGAAAAATCCATACAA
 THRPROVALPHEALAILELYSLYSYASPSERTHRLYSTRPARGLYSLEUVALASPPHEARGGLULEUASNLYSARGTHRGLNASPPHETRPGLUVAL
 2301 TACTCCAGTATTTGCCATAAAGAAAAAAGACAGTACTTAAATGGAGAAAAATTAGTAGATTTTCAGAGAACTTAATAAGAGAACTCAAGACTTCTGGGAAGTT
 GLNLEUGLYILEPROHISPROALAGLYLEULYSLSLYSLYSSERVALTHRVALLEUASPVVALGLYASPALATYRPHESERVALPROLEUASPGLUASPPHE
 2401 CAATTAGGAATACCACATCCCGCAGGGTTAAAAAAGAAAAAATCAGTAAACAGTACAGTGGATGGGTGATGCATATTTTCAGTTCCCTTAGATGAAGACT
 ARGLYSTYRTHRALAPHETHRILEPROSERILEASNANGLUTHRPROGLYILEARGTYRGLNTRYASNVALLEUPROGLNGLYTRPLYSGLYSERPRO
 2501 TCAGGAAGTATACTGCATTTACCATACCTAGTATAAACAAATGAGACACACAGGGATTAGATATCAGTACAATGTGCTTCCACAGGGATGGAAAGGATCACCC
 ALAILEPHEGLNSERSERMETTTHRLYSILELEUGLUPROPHEARGLYSGLNASNPROASPILEVALILETYRGLNTRYMETASPLEUTYRVALGLY
 2601 AGCAATATTCCAAAGTAGCATGACAAAAATCTTAGAGCCTTTAGAAAAACAAAAATCCAGACATAGTTATCTATCAATACATGGATGATTTGTATGTAGGA
 SERASPLEUGLUILEGLYGLNHIISARGTHRLYSILEGLUGLUEUARGGLNHIISLEULEUARGTRPGLYLEUTHRTHRPROASPLYSLSHISGLNLYSGLU
 2701 TCTGACTTAGAAAATAGGGCAGCATAGAACAAAAATAGAGGAGCTGAGACAACATCTGTTGAGGTGGGGACTTACCACACCCAGACAAAAAACATCAGAAAG
 PROPROPHELEUITRPMETGLYTYRGLULEUHIISPROASPLYSYTRPTHRVALGLNPROILEVALLEUPROGLULYSASPSERTRPTHRVALASNASPILE
 2801 AACCTCCATTCTTTGGATGGGTTATGAACTCCATCTCTGATAAATGGACAGTACAGCCATATAGTGTCTGCCAGAAAAAAGACAGCTGGACTGTCAATGACAT
 GLNLYSLEUVALGLYLYSLEUASNTRPALASERGLNILETYRPROGLYILELYSVALARGGLNLEUCYSLYSLEULEUARGGLYTHRLYSALALEUTHR
 2901 ACAGAAGTTAGTGGGGAATTTGAAATGGGCAAGTCAAAATTTACCCAGGGATTAAAGTAAGGCAATATATGTAACCTCTTAGAGGAACCAAGCAGCTAACAT

3001 G L U V A L I L E P R O L E U T H R G L U G L U A L A G L U L E U G L U L E U A L A G L U A S N A R G G L U I L E L E U L Y S G L U P R O V A L H I S G L Y V A L T Y R T Y R A S P P R O S E R L Y S A S P
G A A G T A A T A C C A C T A A C A G A A G A G C A G A G C T A G A A C T G C C A G A A A C A G A G A G A T T C T A A A G A A C C A G T A C A T G G A G T G T A T T A T G A C C C A T C A A A G
LEU ILEALAGLU ILEGLNLYSGNLGYLGNLTPRTPHRTYRGLNILETYRGLNGLUPROPHELYASNLLEULYSTHRGLYLYSTYRALAARGMET
3101 A C T T A A T A G C A G A A A T A C A G A A G C A G G G C A A G G C C A A T G G A C A T A T C A A A T T A T C A A G A G C C A T T T A A A A C T G A A A C A G G A A A A T A T G C A A G A A T
ARGGLYALAHISTHRASNAPVALLYSGNLNLEUTHRGLUALAVALGLNLYSILETHRTHRGLUSERILEVALILETRPGLYLYSTHRPROLYSPHELYS
3201 G A G G G G T G C C C A C A C T A A T G A T G T A A A C A A T T A A C A G A G G C A G T G C A A A A A A T A A C C A C A G A A G C A T A G T A A T A T G G G A A A G A C T C C T A A A T T T A A A
LEUPROILEGLNLYSGLUTHRTPGLUTHRTPHRTYRGLUVALA THRTPHRTYRGLUVALA THRTPHRTYRGLUVALA THRTPHRTYRGLUVALA THRTPHRTYRGLUVALA THRTPHRTYRGLUVALA THRTPHRTYRGLUVALA
3301 C T G C C C C A T A C A A A A G G A A A C A T G G G A A A C A T G G T G G A C A G A G T A T T G G C A A G C C A C C T G G A T T C C T G A T T G G G A G T T T G T C A A T A C C C C T C C T T T A G T G A
LEUTRPTYRGLNLEUGLULYSGLUPROILEVALGLYALAGLUTHRPHEITYRVALASPLVALAALAASNARGGLUTHRARGLEUGLYLYSALAGLYTYR
3401 A A C T A T G G T A C C A G T T A G A G A A A G A C C C A T A G T A G G A G C A G A A A C T T T C T A T G T A G A T G G G C A G C T A A C A G G G A G A C T A G A T T A G G A A A A G C A G G A T A
VALTHRASNLYSGLYARGGLNLYSVALVALPROLEUTHRASNTHRTHRASNGLNLYSTHRGLULEUGLNALAILETYRLEUALALEUGLNASPSERGLY
3501 T G T T A C T A A C A A A G G A A G A C A A A A G G T T G T C C C C C T A A C T A A C A C A C A A A T C A G A A G A C T G A G T T A C A A G C A A T T T A T C T A G C T T T G C A G G A T T C G G G A
LEUGLUALASNILEVALTHRASPSEGLNTYRALALEUGLYILEILEGLNALAGLNPROASPGLUSERGLUSERGLULEUVALASNLNILEILEGLUGLN
3601 T T A G A A G T A A A C A T A G T A A C A G A C T C A C A A T A T G C A T T A G G A A T C A T T C A A G C A C A C A A C A A G T G A A T C A G A G T T A G T C A A T C A A A T A A T A G A G C
LEU ILELYSLYSGLULYSVALTYRLEUALATRPVALPROALAHISLYSGLYILEGLYGLYASNLUGLNLVALASPLYSLEUVALSERALAGLYILEARG
3701 A G T T T A A T A A A A A A G A A A A G G T C T A T C T G G C A T G G G T A C C A G C A C A C A A A G G A A T T G G A G G A A T G A A C A A G T A G A T A A A T T A G T C A G T G C T G G A A T C A G
LYSILELEUPHELEUASPGLYLEASPLYSALAGLNASPGLUHIISGLULYSTYRHISSERASNTRPARGALAMETALASERASPPHEASNLEUPPRO
3801 G A A A A T A C T A T T T T T A G A T G G A A T A G A T A A G C C C A A G A T G A A C A T G A G A A A T A T C A C A G T A A T T G G A G A G C A A T G G C T A G T G A T T T A A C C T T G C C A C C T
VALVALALAYSGLUILEVALALASERCYSASP...YSCYSGNLLEULYSGLYGLUALAMETHISGLYGLNVALASPCYSSERPROGLYILETRPGLNLEUASP
3901 G T A G T A G C A A A A A A A T A G T A G C C A G C T G T G A T A A A T G T C A G C T A A A A G G A G A A G C C A T G C A T G G A C A A G T A G A C T G T A G T C C A G G A A T A T G G C A A C T A G

Fig.2e.

CYS^{THR}HI^SLEUGLY^SVAL^ILELEUVAL^AVAL^HIS^VAL^ALA^SERG^LY^TYR^ILEGL^UAL^AGL^UVAL^ILE^PRO^AL^AGL^UTHR^GL^YGL^NGL^UTHR^A
 4001 ATTGTACACATTAGAGGAAAGTTATCTGGTAGCAGTTCATGTAGCCAGTGGATATA^TAGAAAGCAGAAGTTATTCAGCAGAAACAGGGCAGGAAAC[†]
 ALAT^YRP^HE^LE^UL^YS^{LEU}AL^AGL^YARG^TRP^PRO^VALL^YS^{THR}ILE^HIS^{THR}ASP^SANGL^YSER^ASNP^HE^{THR}SER^AL^ATHR^VALL^YS^AL^AAL^AC^YS[†]
 4101 AGCATATTTCTTTTAA^AATAGCAGGAAGATGGCCAGTAA^{AAA}ACAATAC^AAGACAA^TTTCCAGCAATTTCCACAGTGTACGGTTAAGGCCGCTGT[†]
 TRP^TRP^AL^AGL^YILE^LY^SGL^NGL^UP^HE^GL^YILE^PRO^TYR^ASN^PROGL^NSER^GLNGL^YVAL^{VAL}GL^USER^MET^{ASN}L^YSG^LULE^UL^YS^YILE^ILE^GL^YGL^N
 4201 TGGTGGCGGGGAATCAAGCAGGAATTTGGAAATCCCTACAAATCCCAAGTCAAGGAGTAGTAGAATCTATGAATAAAGAAATTAAGAA^{AAA}ATTATAGGA^C
 VAL^ARG^ASP^{GL}NAL^AGL^UH^IS^{LEU}L^YTHR^AL^AVAL^{GL}N^MET^AL^AVAL^PHE^ILE^HIS^{ASN}P^HE^LY^SARG^LY^SGL^YGL^YILE^GL^YGL^YY^TYR^SER^AL^AGL^Y
 4301 AGGTAAGAGTCAGGCTGAACATCTTAAGACAGCAGTACAAATGGCAGTATTCATCCACAATTTAAAGAAAGGGGGGATTTGGGGGTACAGTGCAGG[†]
 GLU^{ARG}ILE^VAL^{ASP}ILE^ILE^AL^ATHR^{ASP}ILE^GLN^{THR}L^YSG^LULE^ULNGL^YSG^LNILE^THR^LY^SILE^GLN^{ASP}N^PH^EARG^{VAL}T^YR^YARG^{ASP}SER[†]
 4401 GGAAAGAAATAGACATAATAGCAACAGACATACAACTAAAGAAATTA^{CA}AAAA^{TT}CA^{AAA}AA^{TT}TC^{GG}GT^{TT}TAT^TACAGGGACAG^C
 ARG^{ASN}PRO^{LEU}TRP^LY^SGL^YPRO^{AL}L^YS^{LEU}LEU^{TRP}L^YSG^LY^{GL}U^{GL}Y^{AL}VAL^{VAL}ILE^GLN^{ASP}ASN^SER^{ASP}ILE^LY^SVAL^{VAL}PRO^{ARG}ARG^LY^S
 4501 AGAAATCCA^{CT}TTGGAAAGGACCAAGAAAGCTCCTCTGGAAAGGTGAAGGGGCAGTAGTAA^TACAAGA^TAAATAGTGACATA^{AAA}AGTAGTGCCAAAGAAAGAA^G
 MET^{GL}U^{ASN}ARG^{TRP}GL^NVAL^{MET}ILE^VAL^{TRP}GL^NVAL^{ASP}ARG^{MET}ARG^{ILE}ARG^{THR}TRP^LY^SSER^{LEU}VAL^LY^{SH}IS[†]
 4601 AAGCAAAGATCATAGGGA^TTATGGAAACAGATGGCAGGTGATGATTGTGGCAAGTAGACAGGATGAGGATTAGAA^{CA}TGGAA^{AA}AGTTTAGTAA^{AA}AC[†]
 HIS^{MET}Y^RVAL^{SER}GL^YL^SAL^AARG^{GL}Y^{TRP}P^HE^{TYR}ARG^HIS^{HS}IS^{TYR}GL^USER^{PRO}HIS^{PRO}ARG^{ILE}SER^{SER}GL^UVAL^{HS}IS^{ILE}PRO^{LEU}GL^Y
 4701 ACCATATGATGTTTCAGGGAAAGCTAGGGGATGGTTTATAGACATCACTATGAAAGGCCCTCATCCAGAATAAGTTCAGAAGTACACATCCCAC[†]TAGG[†]
 ASP^{ALA}AARG^{LEU}VAL^{ILE}THR^{THR}TYR^{TRP}GL^YLEU^HIS^{THR}GL^YGL^UARG^{ASP}TRP^{HS}IS^{LEU}GL^YVAL^{SER}ILE^GL^UTRP^{ARG}L^YS^LY^SARG[†]
 4801 GGATGCTAGATTGGTAATAACAACATATTGGGGTCTGCA^TACAGGAGAGAAAGAGACTGGCA^{CT}CTGGGT[†]CAGGGAGTCTCCA^TAGAA^TGGAGGAAAGAGAGA^G
 TYR^{SER}THR^{GL}N^{VAL}ASP^{PRO}GL^ULEU^{ALA}ASP^{GL}NILE^UILE^HIS^{LEU}TYR^{TRP}P^HE^{ASP}PC^YSP^HESER^{ALA}ILE^{ARG}L^YS^AL^ALEU^{LEU}GL^YHS[†]
 4901 TATAGCACACAAGTAGACCTGAAC^TAGCAGACCAAC^{TA}ATTCATCTGATTA^{CT}TTTACAGACTCTGCTATAGAAAGGCC^{CT}TATAGGA^C

Fig. 2f.

ILE VAL SER PRO ARG CYS GLU TYR GLN ALA GLY HIS ASN LYS VAL GLY SER LEU GLN TYR LEU ALA LEU ILE THR PRO LYS LYS ILE LYS
 5001 ACATAGTTAGCCCTAGGTGGAATATCAAGCAGGACATAACAAGGTAGGATCTCTACAATACTTGGCACATTAACACCAAAAAGATAAAG
 VAL
 ProProLeuProSerValThrLysLeuThrGluAspArgTrpAsnLysProGlnLysThrLysGlyHisSArgGlySerHisThrMetAsnGlyHisSam*
 5101 GCCACCTTTGGCTAGTGTTACGAAAC TGACAGAGGATAGATGGAAACAAGCCCAAGAGACCAAGGGCCACAGAGGGGAGCCACACAATGAATGGACACTAG
 A
 5201 AGCTTTTAGAGGAGCTTAAGAAATGAAGCTGTAGACATTTTCCTAGGATTTGGCTCCATGGCTTAGGGCAACATATCTATGAAACTTATGGGGATACTTG
 A
 5301 GGCAGGAGTGGAAAGCCATAATAAGAAATTCCTGCAACAACCTGCTGTTTATCCATTTTCAGAAATGGGTGTCGACATAGCAGAAATAGGGCTTACTCGACAGAG
 sa
 5401 GAGAGCAAGAAATGGAGCCAGTAGATCCCTAGACTAGAGCCCTGGAAAGCATCCAGGAAGTCAGCCTAAAACTGCTTGTTACCAATTGCTATTGTAAAAAGTG
 sa
 5501 TTGCTTTTCATTGCCAAGTTTGTTCATAACAAGCCCTTAGGCATCTCTATGGCAGGAAGAAGCGGAGACAGCGAGGAGACCTCTCAAGGCAGTCAG
 G
 5601 ACTCATCAAGTTTCTCTATCAAAGCAGTAAGTAGTACATGTAATGCAACCTATACAAATAGCAATAGTAGCATTAGTAGTGGCAATAATAATAGCAATAG
 C (TAA)
 5701 TTGTGTGTCATAGTAATCATAGAAATATAGGAAATATTAAAGACAAAGAAAAATAGACAGGTTAATTGATAGACTAATAGAAAAGAGCAGAAAGACAGTGG
 A
 env
 METARGVALLYSGLULYSTYRGLNHISLEUTRPARGTRPGLYTRPARGTRPGLYTHRMEITLEULEUGLYMETLEUMETILECYSSERALATHRGLULYS
 5801 CAATGAGAGTGAAGGAGAAATATCAGCACTTGTGGAGATGGGGTGGAGATGGGGCACCATGCTCTTGGGATGTTGATGATCTGTAGTGTACAGAAAA
 A
 LEUTRPVALTHRVALTYR TYRGLYVALPROVALTRP LYSGLUALATHR THR THRLEUPHECYSALASERASPALALYSALATYRASPTHRGLUVALHIS
 5901 ATTGTGGGTCACAGTCTATTATGGGGTACCCTGTGTGGAAAGGAAGCAACCACCACCTCTATTTTGTGTCATCAGATGCTAAAGCATATGATACAGAGGTACAT

Fig.2g.

ASNVALTRPALTHRHSALACYSVALPROTHRASPProAsnProGlnGluValValLeuValAsnValThrGluAsnPhEAsnMetTrpLYSAsnAspMet
 6001 AATGTTGGGCCACACATGCCGTGTGTACCCACAGACCCCAACCCACAGAGTAGTATTGGTAAATGTGACAGAAAAATTAAACATGTGGAAAAATGACA
 VALGUGLNMETHISGLUASPILILESERLEUTRASPGLNSERLEULYSProCYSVALLYSEUThRProLEUCYSVALSERLEULYSCYSTHRASP
 6101 TGGTAGAACAGATGCATGAGGATATAATCAGTTTATGGGATCAAAGCCTAAAGCCATGTGTAATAATTAAACCCACCTCTGTGTAGTTTAAAGTGCACCTGA
 LEULYSAsnAspThRASnThRASNSERSERSERGLYARGMETILEMETGLULYSGLYGLUILELYSAsnCYSSERPHEAsnILESERThRSERILEARG
 6201 TTGTGAAGAAATGATACATAACCAATAGTAGTAGCGGGGAGATGATAATGGAGAAAGGAGAGATAAAAAAATGCTCTTTCAATATCAGCAACAAGCATAAGA
 LYS
 GLYLYSVALGlnLYSGLUTYRALAPHEPHETYRLYSLEUASPILILEProILEASpAsnAspThRThRSERTYRThRLEUThRSERCYSAsnThRSERVAL
 6301 GGTAAGGTGCAGAAAGAAATATGCATTTTTTATAAATTGATATAATACCAATAGATAATGATACCTACCACTACGTTGACAAGTTGTAAACCTCAG
 ILEThRGLNALACYSProLYSVALSERPHEGLUPROILEProILEHISTYRCYSALAPROALAGLYPHEALAILLEULYSCYSAsnAsnLYSTHRPHE
 6401 TCATTACACAGGCCTGTCCAAAGGTATCCTTTGAGCCAAATCCCATACATATTGTGCCCCGGCTGGTTTGGCGATTCTAAAAATGTAATAATAAGACGTT
 ASNGLYThRGLYProCYSTHRASnVALSERThRVALGlnCYSTHRHISGLYILEARGProVALVALSERThRGLNLEULYASnGLYSERLEUALA
 6501 CAATGGAAACAGGACCATGTACAAAATGTCAGCACAGTACAAATGTACACATGGAAATAGGCCAGTAGTATCAACTCAACTGCTGTTAAATGGCAGTCTAGCA
 VAL
 GLUGLUGLVALVALILEARGSERALAAsnPhETHRASpASnALALYSTHRILEILEVALGlnLEUAsnGLNSERVALGLUILEAsnCYSTHRARGProASN
 6601 GAAGAAGAGGTAGTAATTAGATCTGCCAAATTCACAGACAATGCTAAAAACCATAATAGTACAGCTGAACCAATCTGTAGAAAATTAATTGTACAAAGACCCCA
 LYS
 ASNAsnThRARGLYSSERILEARGILEGLNARGGLYProGLYARGALAPHEVALThRILEGLYLYSILEGLYAsnMetARGGLNALAHISCYSSAsnILE
 6701 ACAACAATACAGAAAGAAAGTATCCGATATCCAGAGAGGACCCAGGGAGAGCATTTGTTACAAATAGGAAAAATAGGAAAATATGAGACAAGCACAATTGTAAACAT
 AA
 SERARGALALYSTRPAsnAsnThRLEULYSGlnILEASPserLYSLEUARGGLUGLNPHEGLYAsnAsnLYSTHRILEILEPHELYSGLNSERSERGLY
 6801 TAGTAGAGCAAAAATGGAAATAACACCTTTAAACACAGATAGATAGCAAAATTAAGAGAACAAATTTGGAAATAATAAAACAATAATCTTTAAGCAATCCTCAGGA
 ALA
 GLYAspProGLUILEVALThRHISERPHEAsnCYSGLYGLYGLUPHEPHETYRCYSAsnSERThRGLNLEUPHEAsnSERThRTRPPHEAsnSERThRTRP
 6901 GGGGACCCAGAAAATGTAAACGCACAGTTTTTAATTGGAGGGGAAATTTTCTACTGTAAATCAACACAACTGTTTAAATAGTACTTGGTTTAAATAGTACTT

Fig. 2h.

SER THR GLU GLY SER ASN ASN THR GLU GLY SER ASP THR ILE THR LEU PRO CYS ARG ILE LYS GLN PHE ILE ASN MET TRP GLN GLU VAL GLY LYS ALA
 7001 GGAGTACTGAAGGGGTCAAATAACACCTGAAGGAAGTGACACAATCACACTCCCATGCAGAAATAAACAAATTTATAAACATGTGGCAGGAAGTAGGAAAAGC
 MET TYR ALA PRO PRO ILE SER GLY GLN ILE ARG CYS SER SER ASN ILE THR GLY LEU LEU LEU THR ARG ASP GLY GLY ASN ASN ASN GLU SER GLU
 7101 AATGATATGCCCTCCCATCAGCGGACAAAATTAGATGTTTCATCAAAATATTACAGGGCTGCTATTAAACAAGAGATGGTGGTAATAACAACAATGAGTCCGAG
 ILE PHE ARG PRO GLY GLY ASP MET ARG ASP ASN TRP ARG SER GLU LEU TYR LYS VAL VALLYS ILE GLU PRO LEU GLY VAL ALA PRO THR LYS
 7201 ATCTTCAGACCTGGAGGAGGAGATATGAGGGACAATTGGAGAAGTGAATATATAAATATAAAGTAGTAAAAATTGAACCATTAGGAGTAGCACCCACCA
 ALA LYS ARG ARG VAL VAL GLN ARG GLU LYS ARG ALA VAL GLY ILE GLY ALA LEU PHE LEU GLY PHE LEU GLY ALA ALA GLY SER THR MET GLY ALA ALA
 7301 AGGCAAAGAGAGAGTGGTGCAGAGAGAGAAAAGAGCAGTGGGAATAGGAGCTTTGTTCCTTGGGTTCTTGGGAGCAGCAGGAAGCACATATGGGCGCAGC
 SER MET THR LEU THR VAL GLN ALA ARG GLN LEU LEU SER GLY ILE VAL GLN GLN GLN ASN ASN LEU LEU ARG ALA ILE GLU ALA GLN GLN HI SLEU LEU
 7401 GTCAATGACGCTGACGGGTACAGGCCAGACAATTATTGTCGGTATAGTGCAGCAGCAGAGAACAATTGCTGAGGGCTATTGAGGCGCAACAGCATCTGTTG
 GLN LEU THR VAL TRP GLY ILE LYS GLN LEU GLN ALA ARG ILE LEU ALA VAL GLU ARG TYR LEU LYS ASP GLN GLN LEU LEU GLY ILE TRP GLY CYS SER GLY
 7501 CAACTCACAGCTGGGGGCAACAAGCAGCTCAGGCAAGAAATCCTGGCTGTGGAAAGATACCTAAAGGATCAACAGCTCCTGGGATTTGGGGTTGCTCTG
 LYS LEU ILE CYS THR THR ALA VAL PRO TRP ASN ALA SER TRP SER ASN LYS SER LEU GLN ILE TRP ASN ASN MET THR TRP MET GLU TRP ASP ARG
 7601 GAAACTCAATTTGCACCACCTGCTGTGCTTGGAAATGCTAGTTGGAGTAATAAATCTCTGGAACAGATTTGGAATAACATGACCTGGATGGAGTGGGACAG
 GLU ILE ASN ASN TYR THR SER LEU ILE HI S SER LEU ILE GLU GLU SER GLN ASN GLN GLN LYS ASN GLU GLN LEU LEU GLU LEU ASP LYS TRP
 7701 AGAAATTAACAATTACACAAGCTTAATACACTCCTTAATTTGAAGAAATCGCAAAACAGCAGCAAGAAAAGAAATGAACAAGAAATTTATTTGGAATTAGATAAATGG

9/25

0187041

Fig. 2i.

SER PHE
 7801 ALASNLEUTRPASNTRPLEUASNILETHRASNTRPLEUTRPTYRILELYSLEUPHEILEMETILEVALGLYGLYLEUVALGLYLEUARGILEVALPHEALA
 GCAAATTTGTGGAATTGGTTGAACATAACAAATTGGCTGGTATATAAAATTATTCATATGATAGTAGGAGGCTTGGTAGGTTAAGAAATAGTTTTTG
 VAL
 7901 VALLEUSERILEVALASNARGVALARGGLNGLYTYRSERPROLEUSERPHEGLN^{SP}THRHISLEUPROTHRPROARGGLYPROASPARGPROGLUGLYILE
 CTGTACTTTCTATAGTAAATAGAGTTAGGACGGGATATTCACCATATATCGTTTCAGACCCACCTCCCAACCCGAGGGGACCCGACAGGCCCGAAGGAAT
 GLY
 8001 GLUGLUGLUA^GSPGLYGLUARGASPARGASERILEARGLEUVALASNGLYSERLEUALALEUILETRPASPASP^GLEUARGSERLEUCYSLEUPHE
 AGAAGAAGAAGATGGAGAGAGAGACAGACAGATCCATTCGATTAGTGAACGGATCCTTAGCACCTTATCTGGGACGATCTGGCGAGCCCTGTGCTCTCTC
 8101 SERTYRHISARGLEUARGASPLEU^GLEU^GILEVALTHRARGILEVALGLULEU^GLEU^GYARGGGLYTRPGLUALALEULYSTYRTRPASPASNLEULEU
 AGCTACCA^GCGCTTGAGAGACTTACTCTTGATTTGTAAACGAGGATTGTGGAACCTCTGGGACGACGGGGGTGGGAAGCCCTCAAAATATTGGTGGAAATCTCC
 GLN^GTYRTRPSERGLNGLULEULYSASN^GSERALAVALSERLEULEUASNALA^GTHRALAILEALAV^GALAGLUGLYTHRASPARGVALILEGLUVALVAL
 8201 TACAATATTGGAGTCAGGAGCTAAAGAAATAGTGTGTTAGCTTGTCAATGCCACAGCTATAGCAGTAGCTGAGGGGACAGATAGGGTTATAGAAGTAGT
 GNGLYALATYRARGALAILEARGHISILEPROARGARGILEARGGLNGLYLEUGLUARGILELEULEUOC*^EMETGLYGLYLYSTYRPSERLYSSERSER
 8301 ACAAGGAGCTTATAGAGCTATTCCGCCACATACCTAGAAGAATAAGACAGGGCTTGGAAAGGATTTTGCCTATAAGATGGGTGGCAAGTGGTCAAAAAGTAG
 VAL
 8401 VALILEGLYTRP^GPROALAVALARGGLUARGMETARGARGALAGLUPROALAALAASPGLYVALGLYALAALASERARGASPLEUGLULYSHISGLYALA
 TGTGATTGGATGGCTGCTGTAAAGGGAAAGAAATGAGACGAGCTGAGCCAGCAGCAGATGGGGTGGGAGGAGCATCTCGAGACCTAGAAAAACATGGAGCA

Fig.2j.

THR ASP
 ILETHRSESERASNTHRALAAASNASNALAALACYSALATRPLEUGLUAGLUGLUGLYSVALGLYPHEPROVALTHRPROGLNVALPROLEU
 8501 ATCACAAGTAGCAACACAGAGCTAACAAATGCTGCTTGCCCTGGCTAGAACACAGAGGAGGAGAGGTTTCCAGTCACACCTCAGGTACCTT
 ARGPMETTHRTRYRLYSALAALAVALASPLEUSERHTSPHELEULYSGLULYSGLYGLYLEUGLUGLYLEUILEHISSEERGLNARGARGGLNASPIL
 8601 TAAGACCAAAGACTTACAAAGGCAGCTGTAGATCTTAGCCACTTTTAAAAGAAAAGGGGGGACITGGAGGGCTAAATTCACTCCCAACGAAGACAAGATAT
 LEUASPLEUTRPILEITYRHISTRHGLNGLYTYRPHPEPROASPIRPGLENASNTYRTHRPROGLYPROGLYILEARGTYRPROLEUTHRPHEGLYTRPCYS
 8701 CCTTGATCTGTGGATCTACACACACAAAGGCTACTTCCCTGATTGGCAGAACTACACACACAGGACAGGATCAGATATCCACTGACCTTTGGATGGTGC
 TYRLYSLEUVALPROVALGLUPROASPLYSVALGLUGLUALAASNLYSGLYGLYUASNTHRSEERLEUHISSPROVALSERLEUHIISGLYMETASPPRO
 8801 TACAAGCTAGTACCAGTTGAGCCAGATAAGGTAGAAAGAGGCCAACAAAGGAGAGAACACACAGCTTGTACACCCCTGTGAGCCTGCATGGAAATGGATGACC
 GLUARGGLUVALLEUGLUUTRPARGPHEASPSERARGLEUALAPHEHISHISVALALAAARGGLULEUHISSPROGLUTYRPHELYSASNCYSOP*
 8901 CCGAGAGAGAAGTGTAGAGTGGAGGTTTGACAGCCGCTAGCATTTTCACTACAGTGGCCCGAGAGCTGCATCCGGAGTACTTCAAGAACTGCTGATATCG
 9001 AGCTTGCTACAAGGGACTTTCGGCTGGGGACTTTCAGGGGAGGCTGGCCCTGGGCGGGACTGGGAGAGTGGCGAGCCCTCAGATGCTGCAATAAGCAGCT
 9101 GCTTTTGGCTGTACTGGGTCCTCTCTGGTTAGACCAGATCTGAGCCTGGGAGCTCTCTGGCTAACTAGAGAACCCACTGCTTAAAGCCTCAATAAAGCTTG
 9201 CCTTGAGTGCTTC

12/25

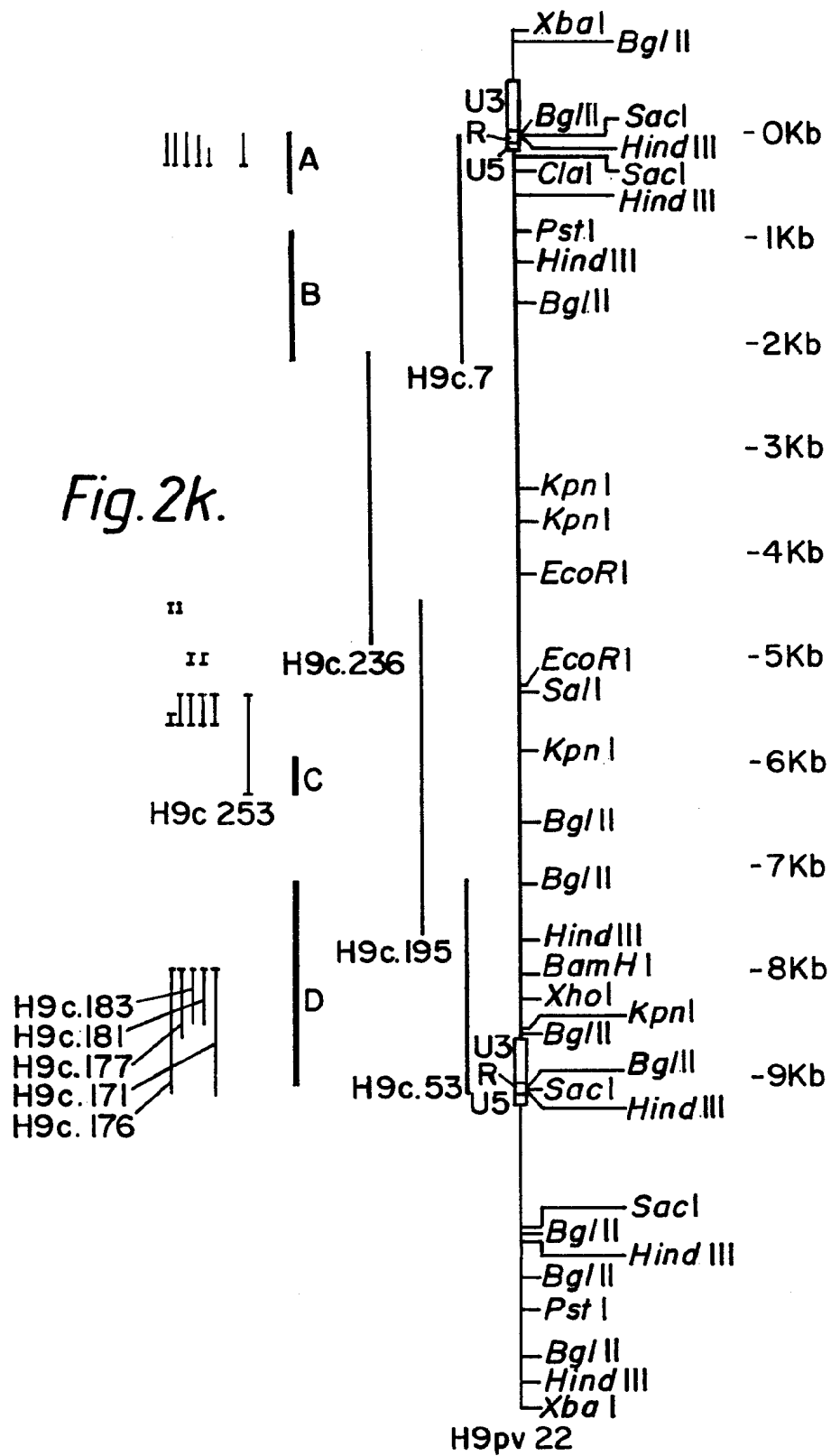
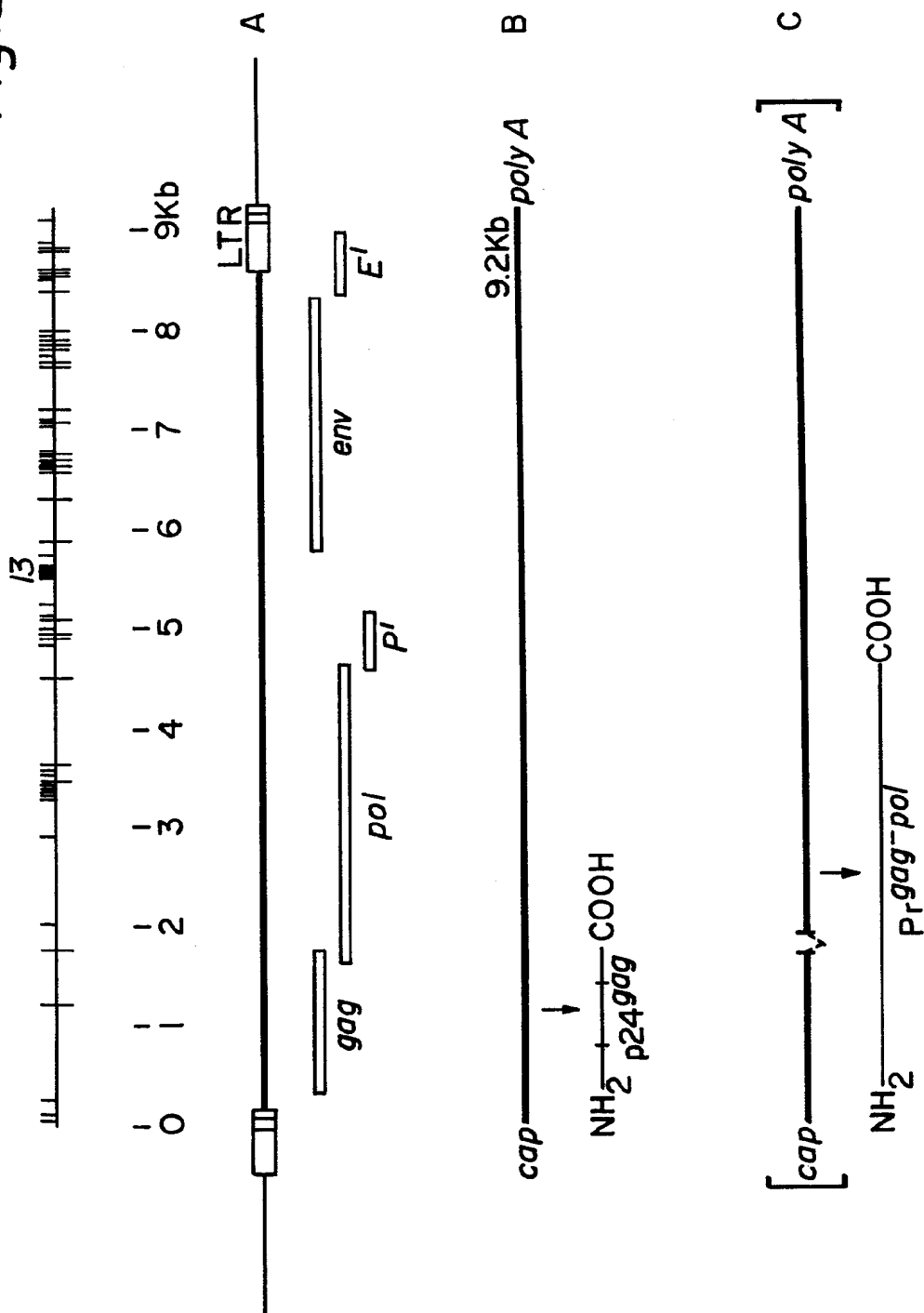
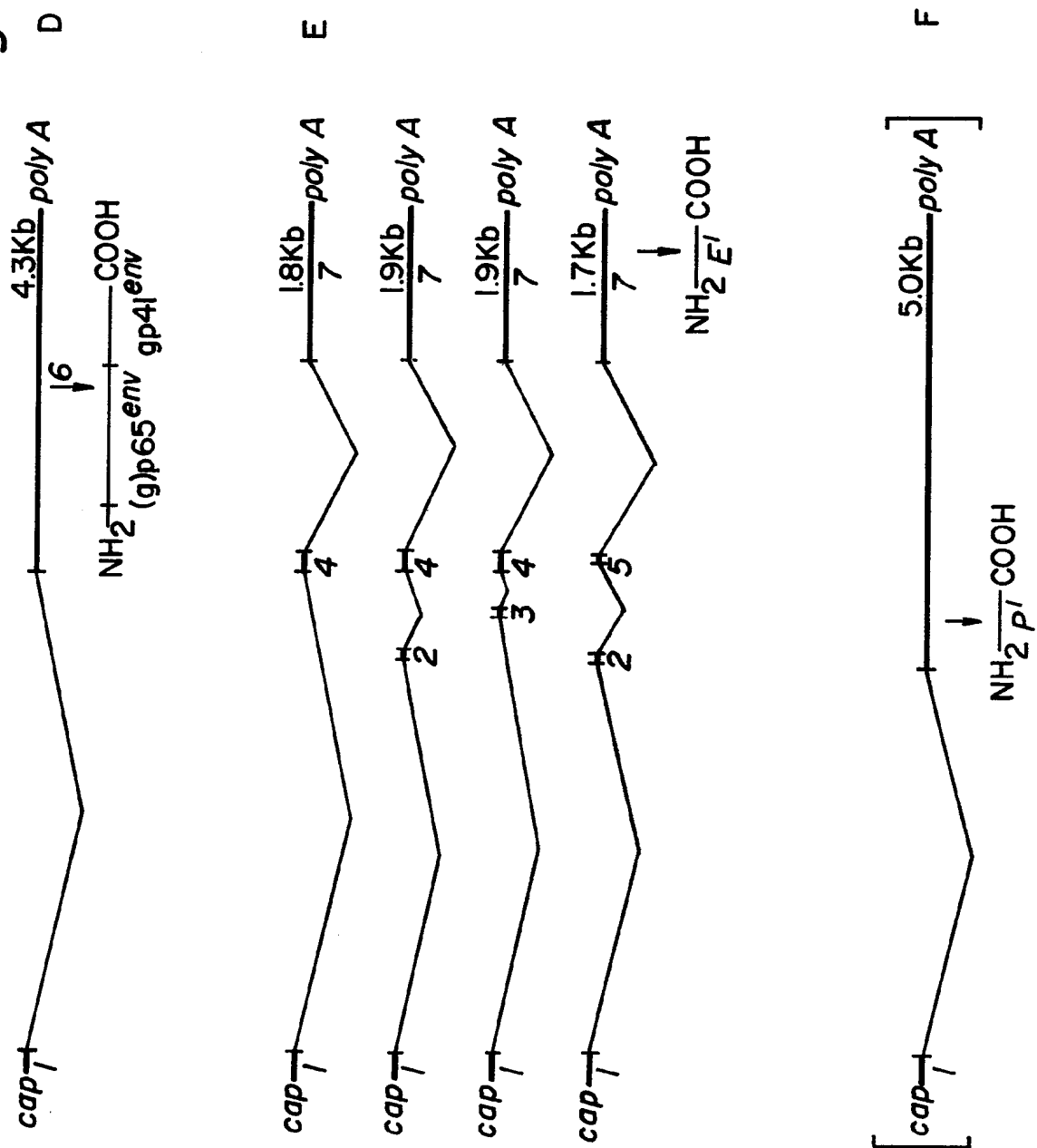


Fig. 21.



14/25

Fig. 21(Cont.)



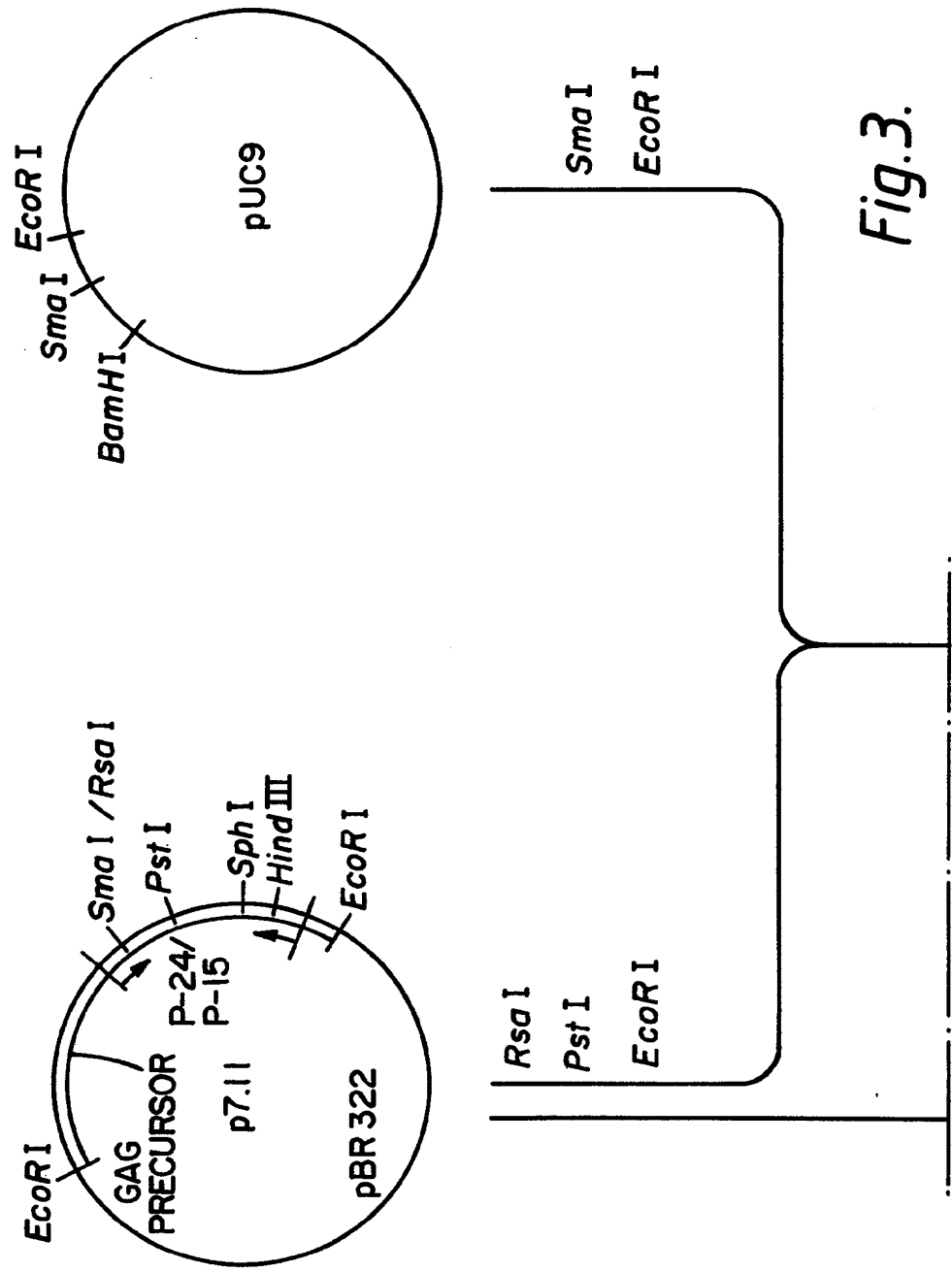


Fig.3.

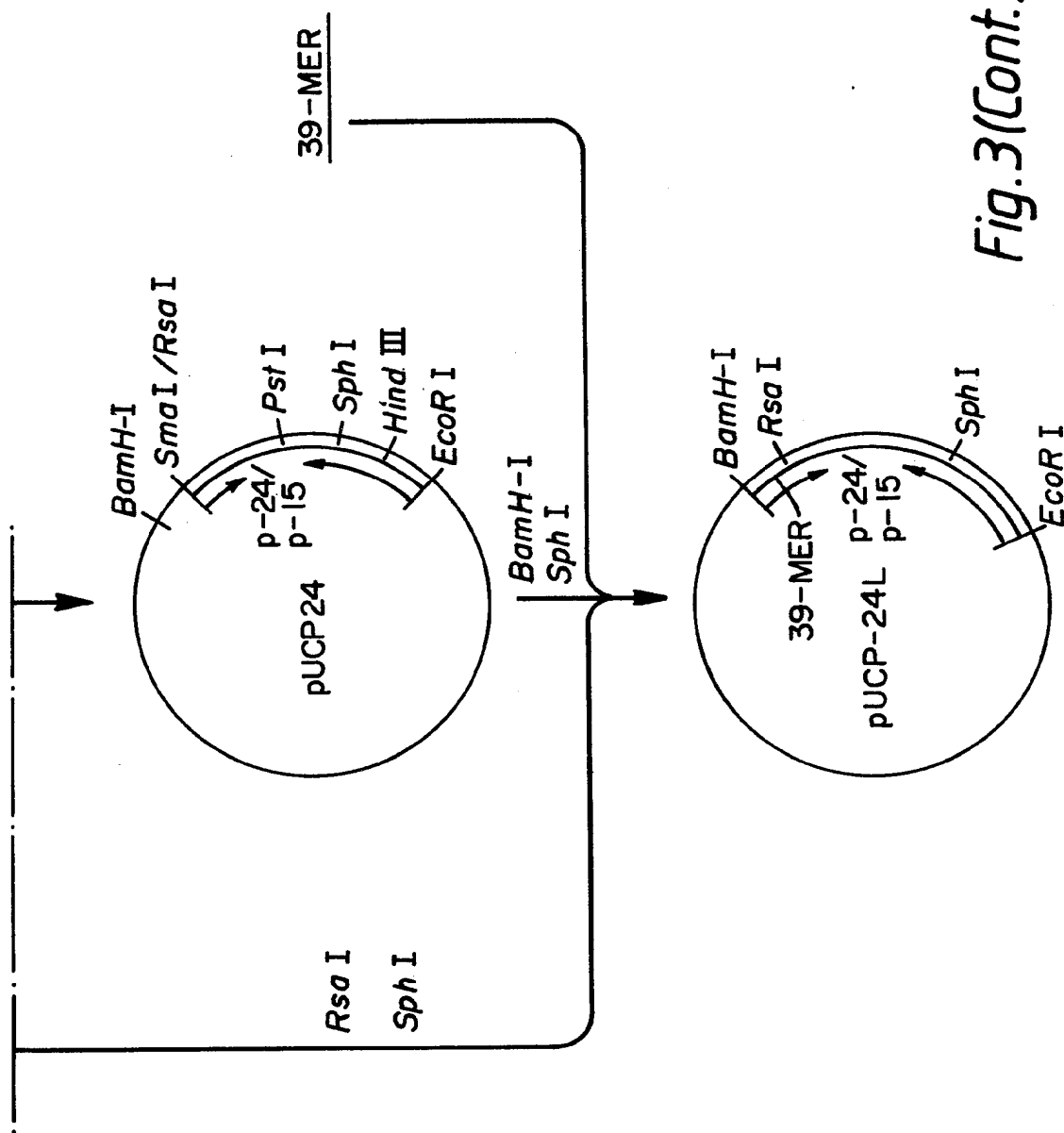
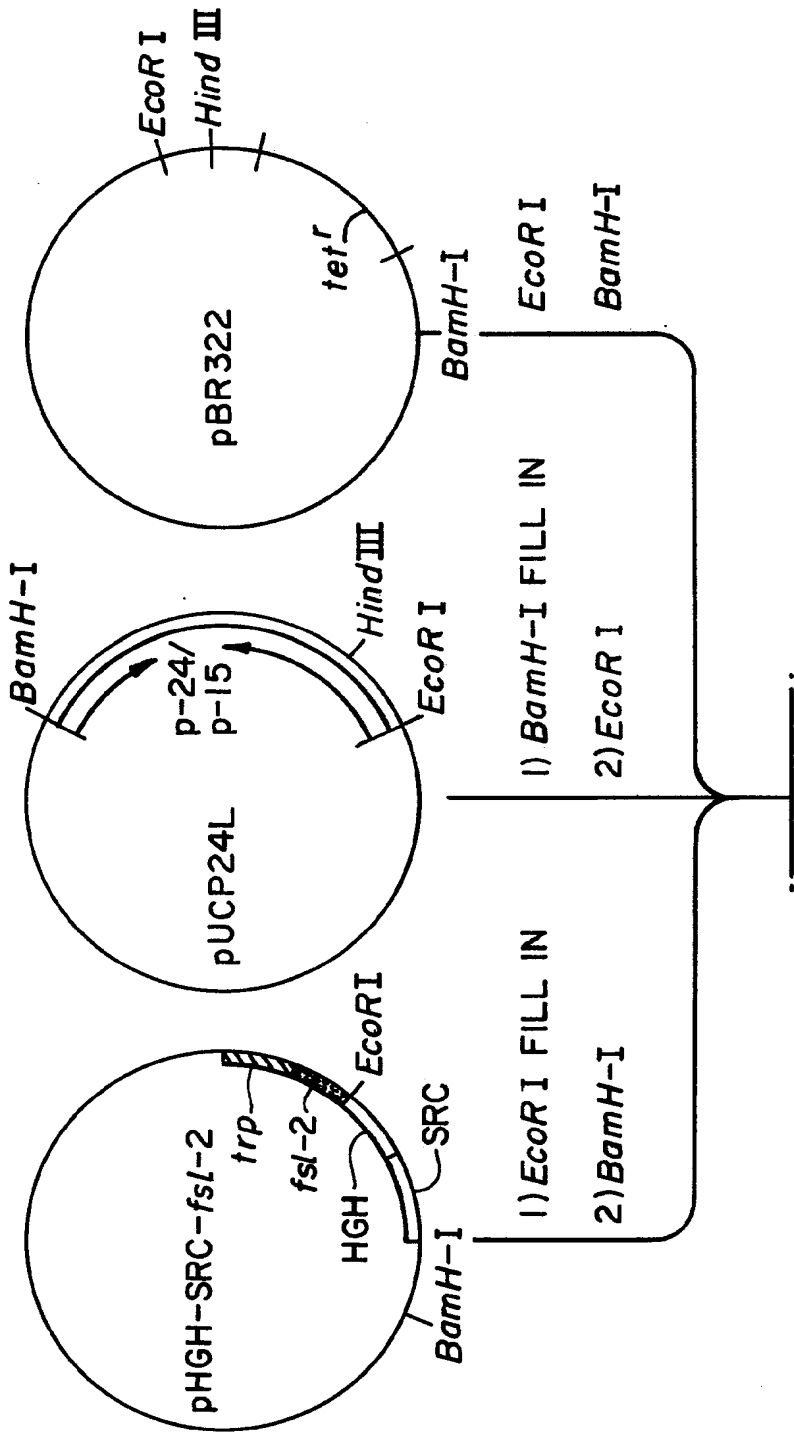
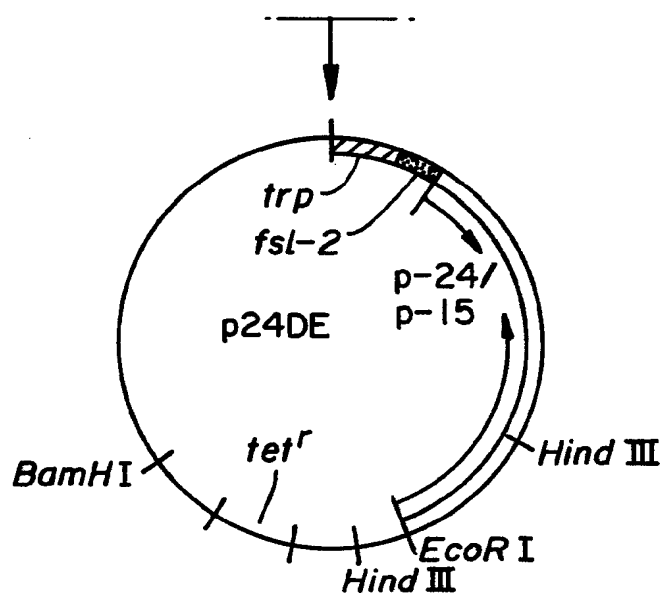
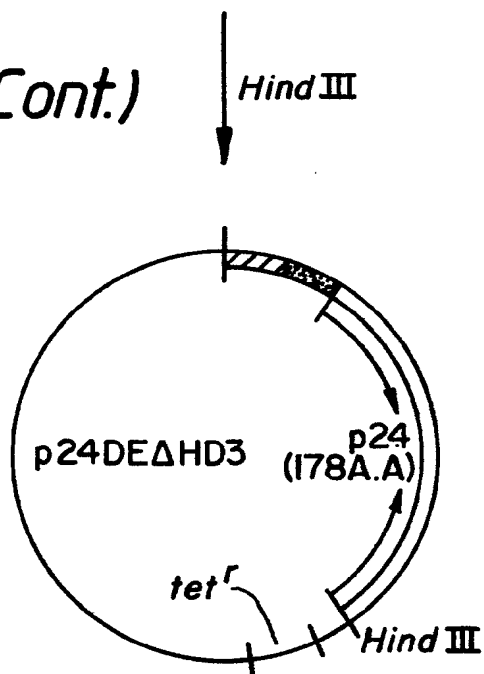


Fig. 3(Cont.)

Fig. 4.

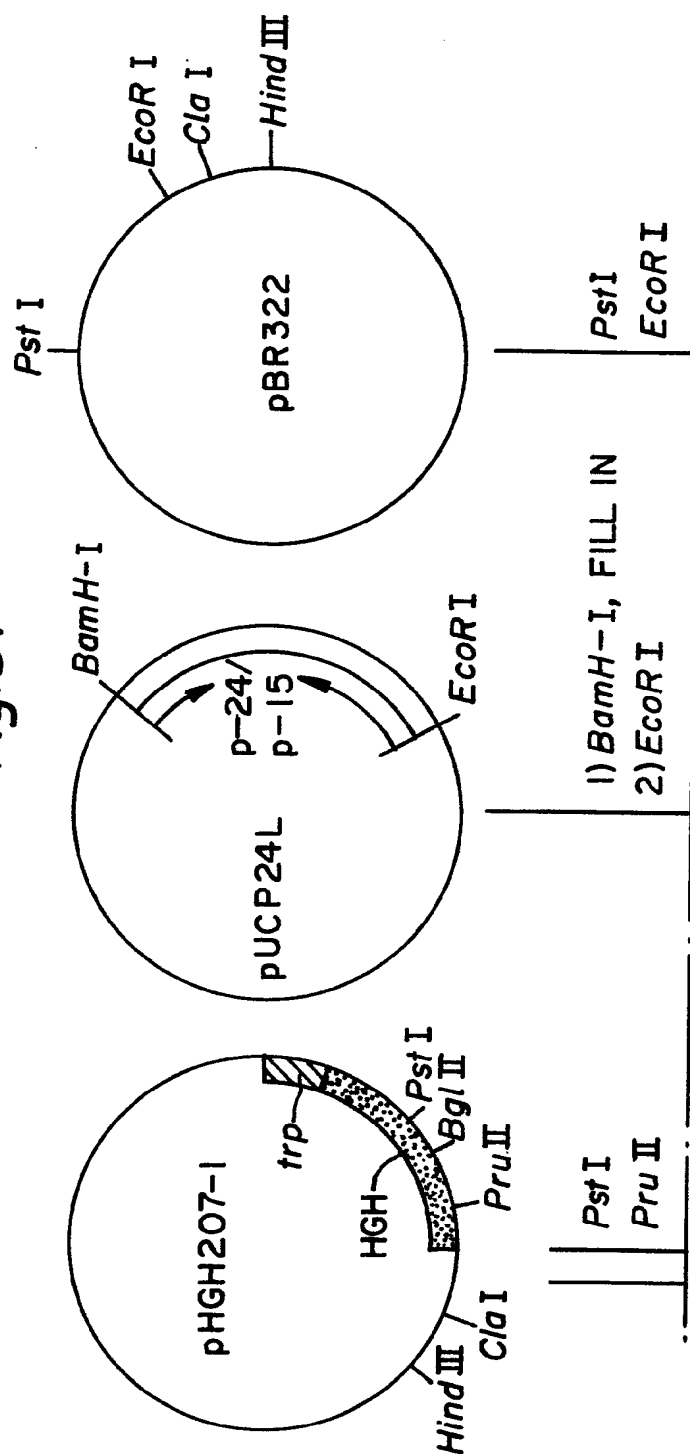


18/25

*Fig.4(Cont.)*

19/25

Fig. 5.



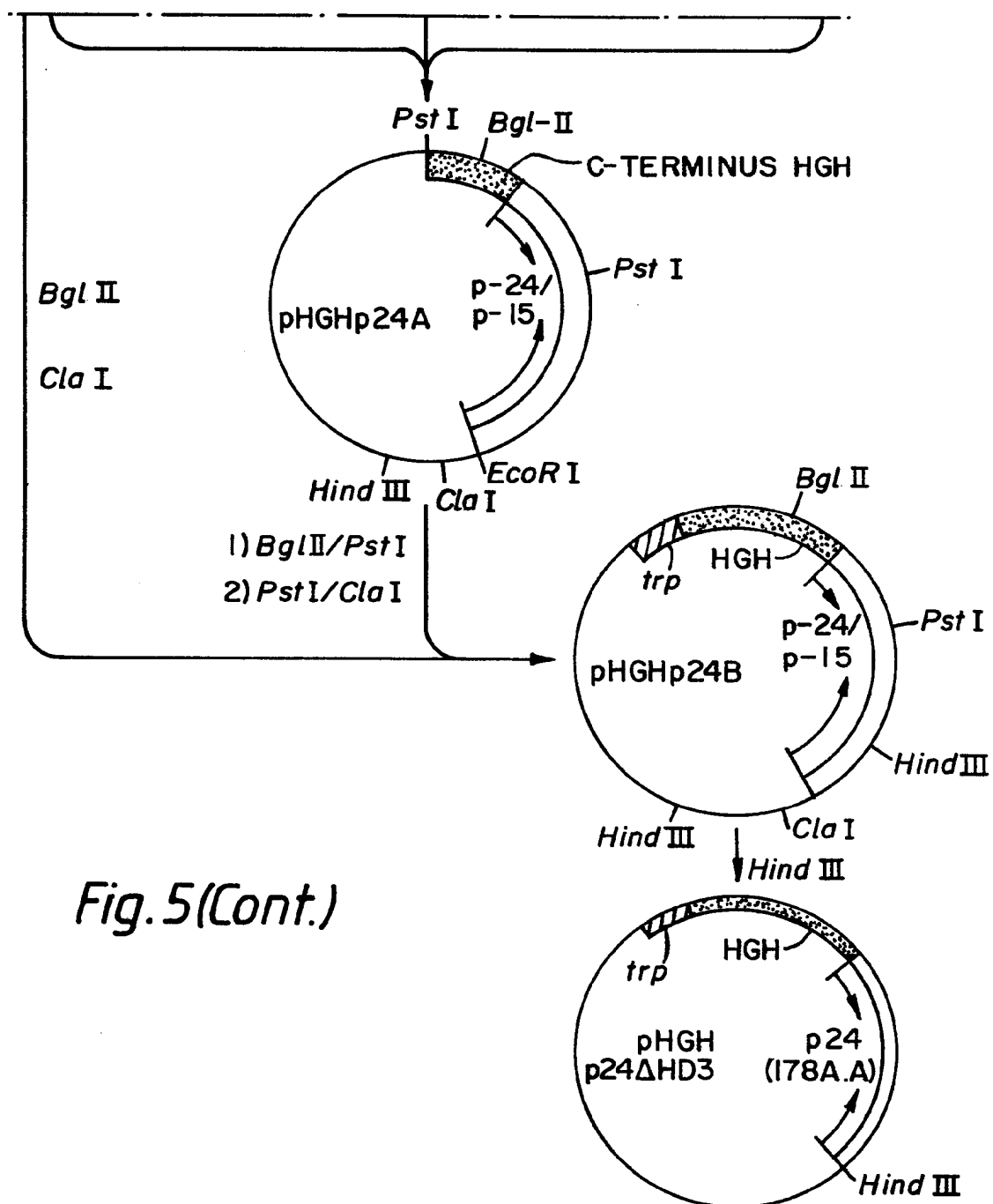


Fig. 5(Cont.)

21/25

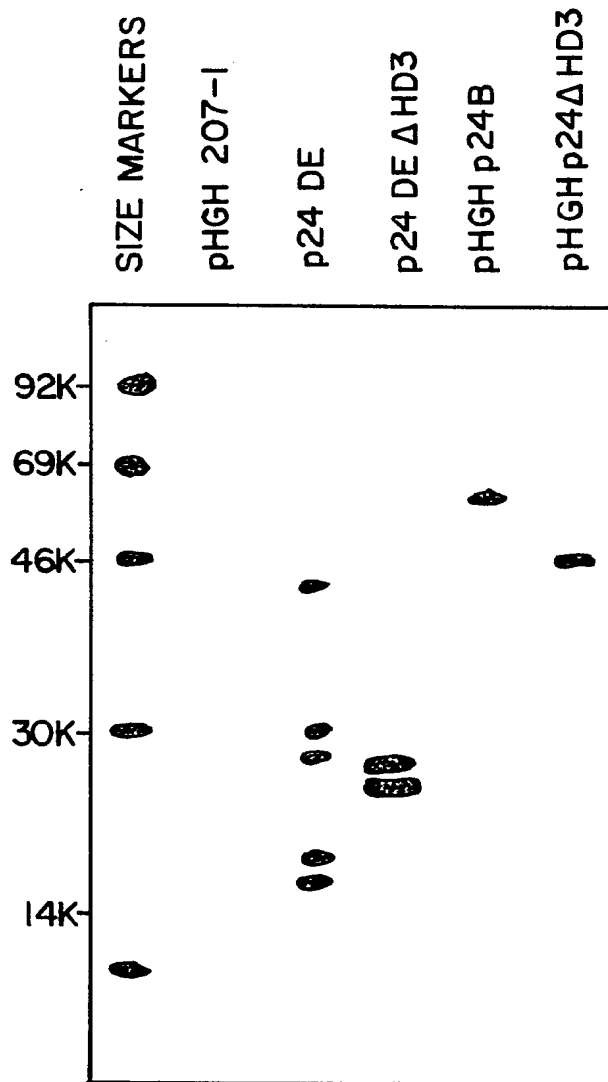
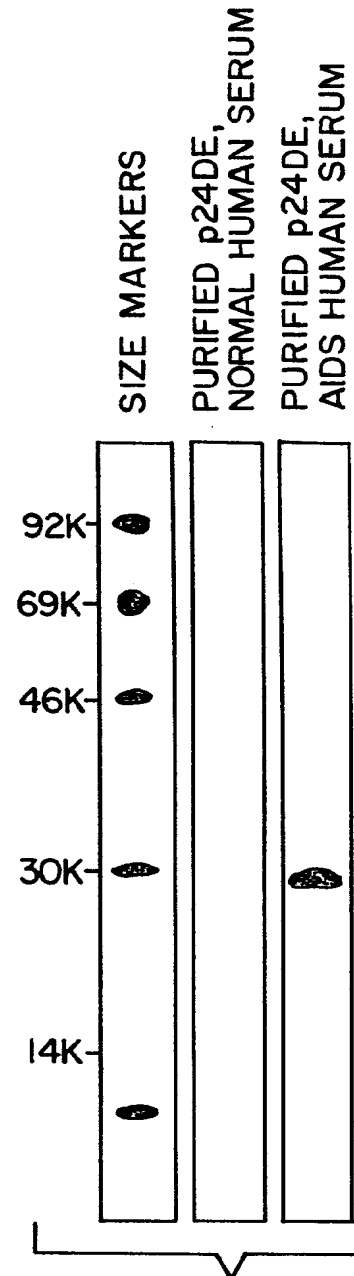
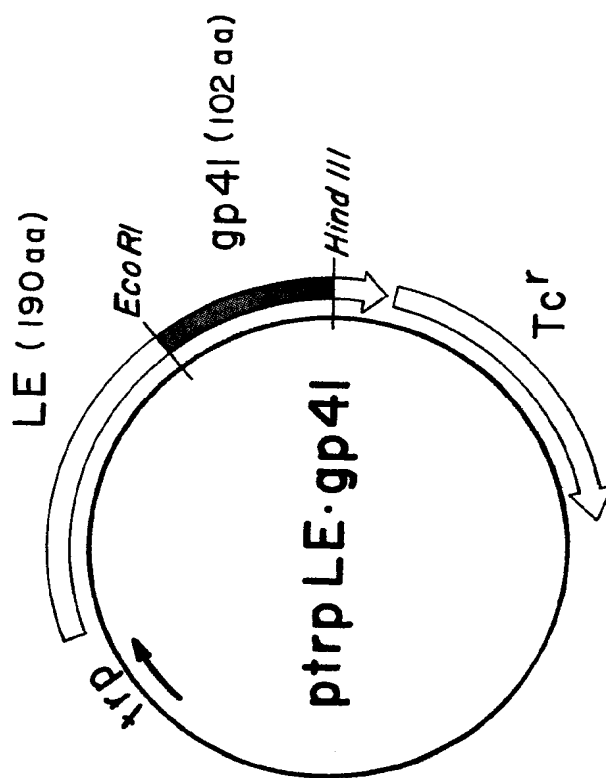
*Fig.6.**Fig.7.*

Fig.8.

A.



B.

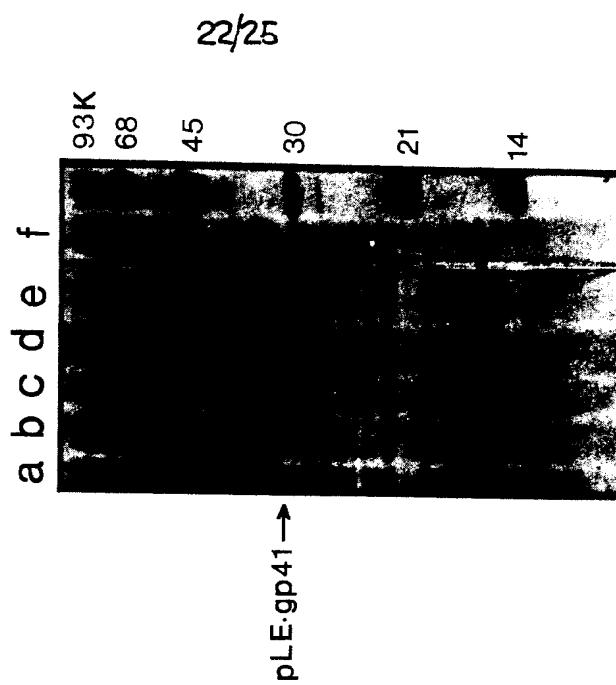
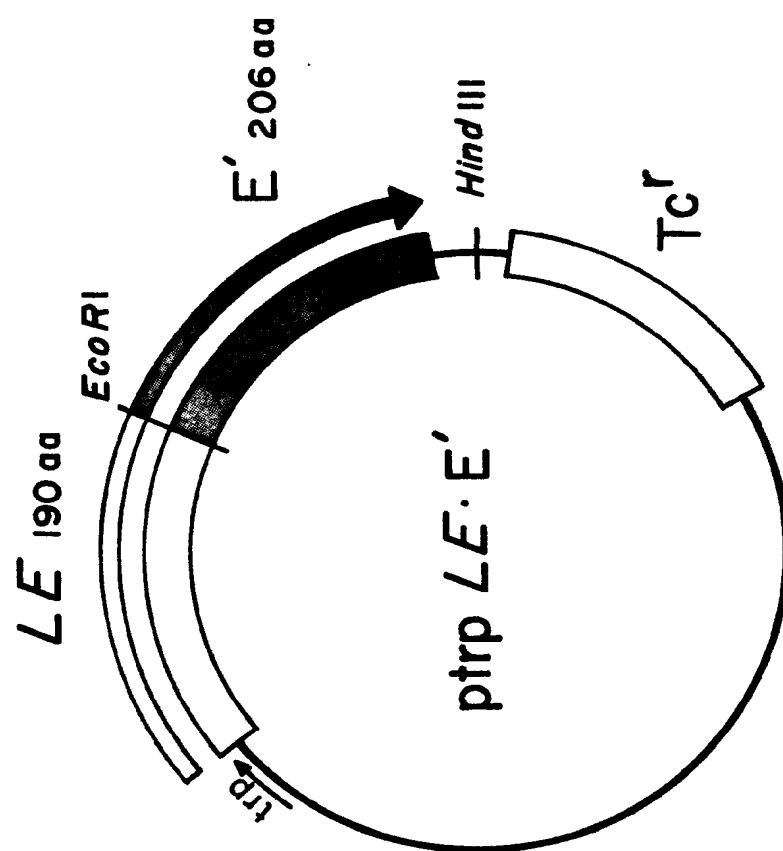
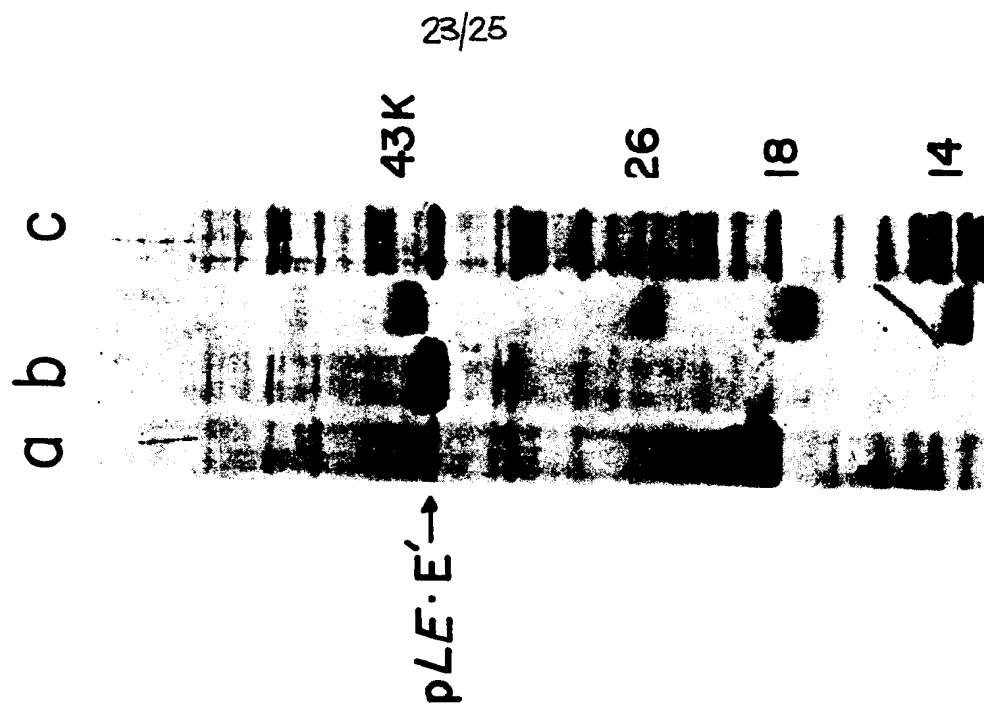


Fig. 9.

A.

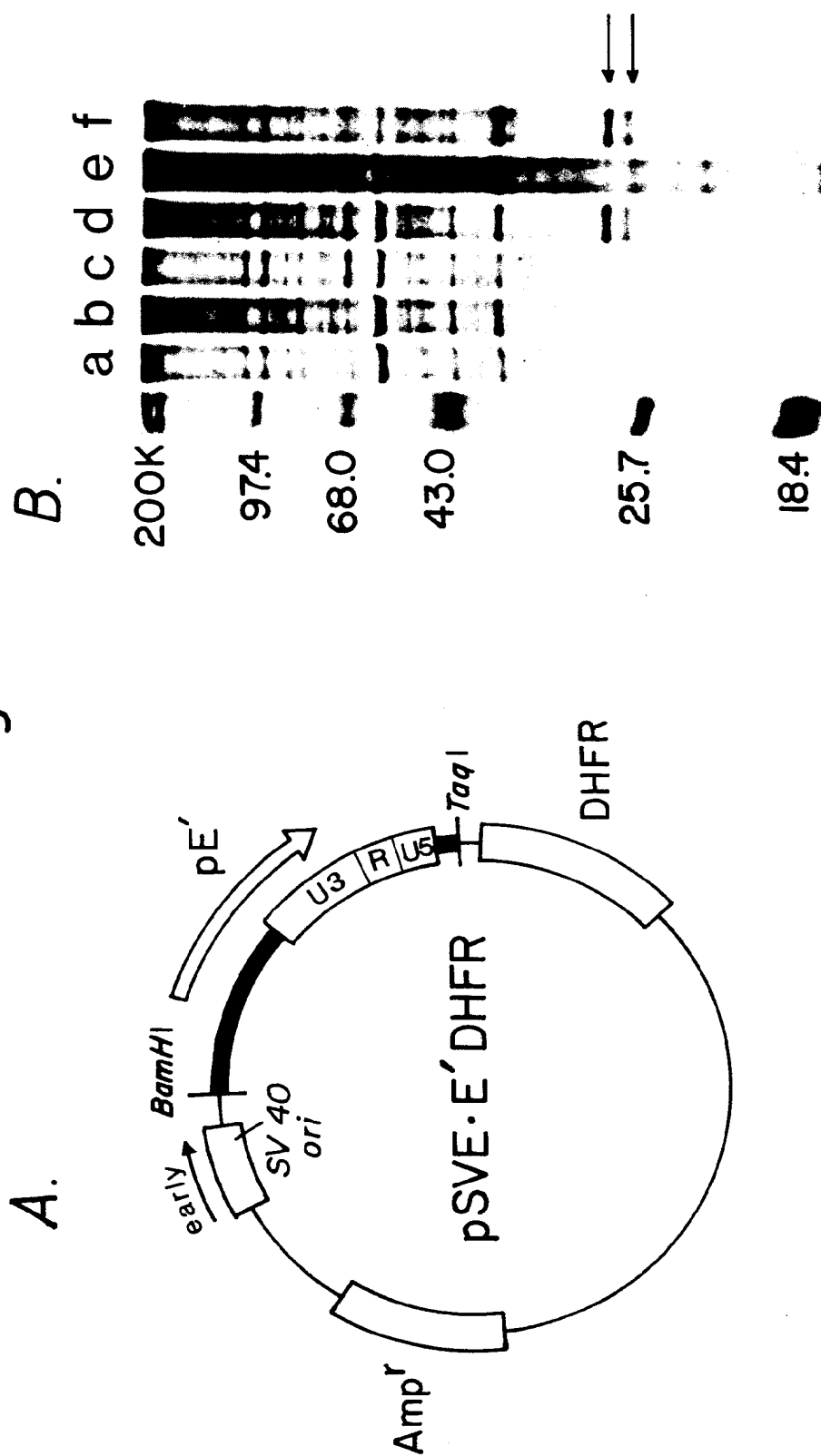


B.



24/25

Fig. 10.



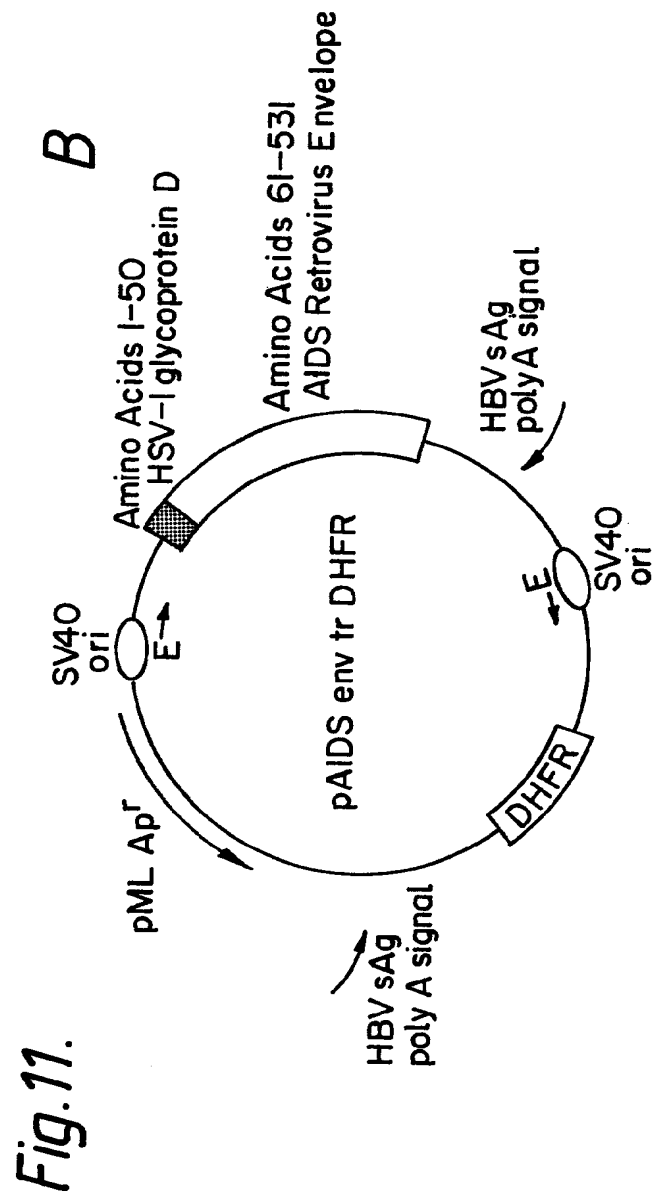
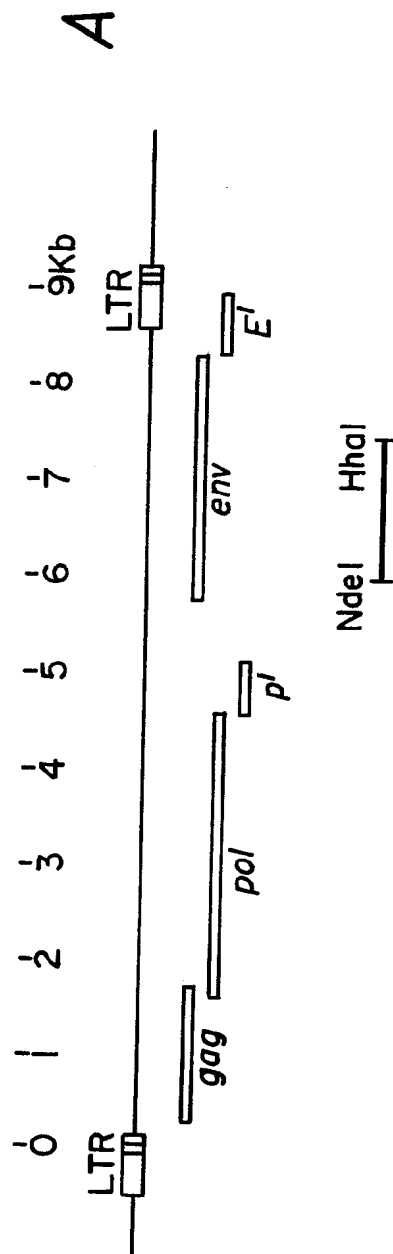


Fig.11.



European Patent
Office

EUROPEAN SEARCH REPORT

0187041

Application number

EP 85 30 9454

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.4)
X	SCIENCE, vol. 224, 4th May 1984, pages 503-505; J. SCHÜPBACH et al.: "Serological analysis of a subgroup of human T-lymphotropic retroviruses (HTLV-III) associated with AIDS" * Whole document *	1-4,9- 11,17, 18,27- 29,38- 41	C 12 N 15/00 A 61 K 39/21 C 12 P 21/02 C 07 K 13/00 C 12 Q 1/70 G 01 N 33/569 C 12 N 1/20 C 12 N 5/00 C 12 N 9/10
X	SCIENCE, vol. 225, 20th July 1984, pages 321-323; V.S. KALYANARAMAN et al.: "Antibodies to the core protein of lymphadenopathy-associated virus (LAV) in patients with AIDS" * Figure 1, lane C *	1,2,9- 11,27- 29,38, 39	
X	SCIENCE, vol. 226, 26th October 1984, pages 447-449; J.E. GROOPMAN et al: "HTLV-III in saliva of people with ADIS-related complex and healthy homosexual men at risk for AIDS" * Figure 2 *	1,2,9- 11,27- 29,38, 39	TECHNICAL FIELDS SEARCHED (Int. Cl.4) C 12 N C 12 P C 12 Q A 61 K
X	SCIENCE, vol. 226, 26th October 1984, pages 453-456; F. BRUN-VEZINET et al.: "Prevalence of antibodies to lymphadenopathy-associated retrovirus in african patients with AIDS" * Figure 1 *	1,2,9- 11,27- 29,38, 39	
The present search report has been drawn up for all claims			
Place of search THE HAGUE		Date of completion of the search 17-03-1986	Examiner CUPIDO M.
<p>CATEGORY OF CITED DOCUMENTS</p> <p>X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document</p> <p>T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons & : member of the same patent family, corresponding document</p>			

0187041



European Patent
Office

EUROPEAN SEARCH REPORT

Application number

EP 85 30 9454

Page 2

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.4)
X	ANNALES DE VIROLOGIE, vol. 135E, 1984, pages 119-134; L. MONTAGNIER et al.: "A new type of retrovirus isolated from patients presenting with lymphadenopathy and acquired immune DEFICIENCY SYNDROME: structural and antigenic relatedness with equine infectious anaemia virus" * Figure 5 *	1,2,9- 11,27- 29,38, 39	
X,P D	--- NATURE, vol. 313, 7th February 1985, pages 450-458; M.A. MUESING et al.: "Nucleic acid structure and expression of the human AIDS/lymphadenopathy retrovirus" * Whole document *	1-64	
X,P	--- WO-A-8 504 903 (UNITED STATES OF AMERICA) * Claims 1-46 *	1-4,9- 11,17, 18,27- 29,38- 41	
X,P	--- EP-A-0 138 667 (INSTITUT PASTEUR) * Claims 5-7 *	1,2,9- 11,27- 29,38, 39	
The present search report has been drawn up for all claims			
Place of search THE HAGUE		Date of completion of the search 17-03-1986	Examiner CUPIDO M.
<p>CATEGORY OF CITED DOCUMENTS</p> <p>X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document</p> <p>T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons & : member of the same patent family, corresponding document</p>			



European Patent
Office

EUROPEAN SEARCH REPORT

0187041

Application number

EP 85 30 9454

Page 3

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.4)
E	EP-A-0 173 529 (UNITED STATES OF AMERICA) * claim 14 * -----	8,9,30	
			TECHNICAL FIELDS SEARCHED (Int. Cl.4)
The present search report has been drawn up for all claims			
Place of search THE HAGUE		Date of completion of the search 17-03-1986	Examiner CUPIDO M.
CATEGORY OF CITED DOCUMENTS			
X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document		T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons & : member of the same patent family, corresponding document	